

PB-216 589

PROCEEDINGS OF NORTHWEST SHELLFISH SANI-
TATION RESEARCH PLANNING CONFERENCE
(1965)

W. J. Beck, et al

Northwest Shellfish Sanitation Laboratory
Gig Harbor, Washington

1966

DISTRIBUTED BY:

NTIS

National Technical Information Service
U. S. DEPARTMENT OF COMMERCE
5285 Port Royal Road, Springfield Va. 22151

ENVIRONMENTAL HEALTH SERIES
Food Protection

PB 216 589

1965
PROCEEDINGS



Northwest Shellfish Sanitation Research Planning Conference

Reproduced by
**NATIONAL TECHNICAL
INFORMATION SERVICE**
U.S. Department of Commerce
Springfield VA 22151

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service

1965
PROCEEDINGS

Northwest Shellfish Sanitation Research Planning Conference

W.J. Beck and J.C. Hoff

Northwest Shellfish Sanitation Laboratory
Gig Harbor, Washington

Details of illustrations in
this document may be better
studied on microfiche.

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
Division of Environmental Engineering and Food Protection
Shellfish Sanitation Branch
1966

Disclaimer Clause

The use of trade names of products in this publication are for example only and do not imply endorsement by the Public Health Service or the U. S. Department of Health, Education and Welfare.

Public Health Service Publication No. 999-FP-6

PREFACE

The annual Northwest Shellfish Sanitation Research Planning Conference has become a cardinal factor in the research program of the Northwest Shellfish Sanitation Laboratory. The success of these conferences has been made by the participation of industry, universities and state and federal agencies, both in this country and Canada. The frank discussions of problems through the past years have aided our efforts immeasurably.

During the past year significant change has evolved, both in the physical facility and direction of research. Several projects have been reoriented to delve in basic problems associated with the sanitary control of shellfish today. As an indication of this change, the reports presented are now being published as a formal Proceedings of Northwest Shellfish Sanitation Research Conference.

Our appreciation is extended to the many persons whose effectual counsel has made these conferences a success throughout the past seven years.

W. J. Beck

CONTENTS

	<u>Page</u>
 PART I. RESEARCH PROGRESS REPORTS	
Summarization of Activities, W. J. Beck	2
Membrane Filter Technics as Applied to Estuarine and Tributary Waters W. J. Beck and G. J. Vasconcelos	3
Bacteriological Studies on Commercial Processing of Olympia Oysters (<u>Ostrea lurida</u>) G. J. Vasconcelos	15
Storage Studies on Shucked Olympia Oysters (<u>Ostrea lurida</u>) T. H. Ericksen	23
Storage Studies on Olympia Oyster Shellstock (<u>Ostrea lurida</u>) G. J. Vasconcelos	32
Antimicrobial Agents in West Coast Shellfish W. Jakubowski	43
Research Activities at Other Public Health Service Shellfish Sanitation Centers C. B. Kelly	54
Preliminary Ecological Studies on the Presence of <u>Clostridium botulinum</u> T. H. Ericksen and W. J. Beck	58
Incidence of <u>Clostridium botulinum</u> on the Pacific Coast M. Eklund	70
Depuration of Poliovirus Polluted Clams O. C. Liu	73
Studies on Bacteriophage Accumulation and Elimination by the Pacific Oyster (<u>Crassostrea gigas</u>) J. C. Hoff, W. Jakubowski, and W. J. Beck	74
Studies on Membrane Filtration of Bacteriophage J. C. Hoff and W. Jakubowski	91

PART II. PROPOSED RESEARCH ACTIVITIES FOR FISCAL YEAR 1966

Introduction	102
Wet Storage Studies	102
Anaerobic Bacteriology	103
Antimicrobial Investigations	104
Virus Research	105
Depuration Studies	105
Commensal Bacteriology	106
Technical Assistance	106

PART III. APPENDIX

Agenda	109
Attendance Roster	111

PART 1

RESEARCH PROGRESS REPORTS

SUMMARIZATION OF ACTIVITIES

W. J. Beck

Within the past year, the Northwest Shellfish Sanitation Laboratory has undergone a period of transition. Changes have been made in the physical plant, personnel and in the types of research projects. Emphasis has been placed in the fields of virology and anaerobic bacteriology as related to shellfish and shellfish growing areas.

In 1964 we published our Proceedings of the Sixth Annual Research Planning Conference. Storage studies now have been made on the major commercially important shellfish on the Pacific Coast except butter clams. A technical report on storage of Pacific oysters, Olympia oysters, Native Littleneck and Manila clams, is now being prepared for publication.

Research has continued on the relationship of bacteriophages to Pacific oysters. This phase of our virology research is now nearing completion. The search for antimicrobial and antiviral agents in shellfish has been initiated. A report on several technics which have been used in these fields of research is presented in these proceedings. Preliminary investigations on methods of enumeration and identification of other viruses as related to shellfish are now being made.

An ecological study of anaerobic bacteria in estuarine areas has been initiated. This study has now passed the preliminary stage. Although the study on wet storage of shellfish is not yet in progress, preliminary plans have been made and a proposed project is now being considered. The first phase of this study will be completed by July, 1966.

Activities in technical assistance have been varied throughout the year. Laboratory evaluations included two local laboratories. A cooperative program with the College of Fisheries, University of Washington, for the training of Ph. D. candidates in shellfish sanitation is now under way. Two reports were included in the 1964 National Shellfish Sanitation Workshop. These papers included research on storage of Pacific oysters and the bacteriological criteria for shellfish growing areas. Much effort has gone into the evaluation of potential sites for the proposed Shellfish Sanitation Research Center on the Pacific Coast. Background data has been obtained on various sites in the States of Oregon and Washington.

We at the Northwest Shellfish Sanitation Laboratory believe that we are merely the prelude to the expansion of research in shellfish sanitation on the West Coast. Through the cooperation shown by federal and state agencies, universities, and industry, we can soon attempt many facets of research in this field that we could not consider in the past.

MEMBRANE FILTER TECHNIQS AS APPLIED TO ESTUARINE AND TRIBUTARY STREAMS

W. J. Beck and G. J. Vasconcelos

INTRODUCTION

The Third Edition of APHA Recommended Procedures for the Bacteriological Examination of Sea Water and Shellfish (1962) described a membrane filter technic as a tentative method in evaluating the sanitary quality of sea water. Certain restrictions were placed on this technic until further evaluations could be made. In recent years McCarthy (1961) and Taylor (1955) have proposed modifications for the use of the membrane filter technic in the enumeration of coliforms in both estuarine and fresh water samples. Geldreich, Clark, Huff and Rost (1965) have proposed a m-FC Broth for enumeration of fecal coliform organisms by the membrane filter technic.

When a large number of water samples are required in the evaluation of growing areas, the MF technic would be valuable to the control laboratory. If reliable results could be assured, the savings in time, personnel and cost per test would be considered an asset.

A series of studies was initiated in 1961 to evaluate the reliability and selectivity of several of the MF technics available for enumeration of coliform organisms.

In 1965 a technic suggested by Geldreich, Clark, Huff and Rost (1965) for the enumeration of fecal coliform organisms by the MF method was incorporated into the study.

Samples included in the study consisted of estuarine water from a shellfish growing area and fresh water from the same vicinity. Thus factors such as ambient temperature, rainfall and subsequent runoff would be in the same general range.

MATERIALS AND METHODS

Samples:

The fresh water samples were collected in the general areas described by Kelly, Beck, Presnell and Zobel (1962). Sea water samples were from both high and low tide stages at the salt water intake of the Shellfish Sanitation Laboratory. Samples were taken through a four-year period during all seasons.

Multitube Method:

All samples were examined by the 5 tube multitube method according to APHA Recommended Procedures for the Bacteriological Examination of Sea Water and Shellfish (Third Edition, 1962). Confirmation for fecal coliform organisms was made in EC medium incubated in a water bath at 44.5 $\pm$ 0.2 C.

MF Technics Used for Enumeration of Coliform Organisms:

The three MF technics for the enumeration of coliform organisms included the M-Endo (Standard Methods); McCarthy MF and a Modified MacConkey MF technic.

1. M-Endo (Standard Methods)

This method is the one described in APHA Recommended Procedures for the Bacteriological Examination of Sea Water and Shellfish (Third Edition, 1962). The medium was prepared as directed by the manufacturer except that it was prepared fresh each day. All samples tested by this technic were incubated in a saturated atmosphere at 35 $\pm$ 0.5 C for 22 $\pm$ 2 hours.

2. MacConkey MF Technic

The MacConkey MF technic proposed by Taylor (1955) consisted of two steps. The filtered sample was first incubated on a yeastrel medium for two hours at 35 C. After this enrichment period the filter was transferred to a Modified MacConkey Medium for incubation at 35 C for 20 hours. The original formula consisted of English ingredients exclusively. Comparative tests at this station between products available in this country and foreign products failed to reveal any preference for either type of ingredient. Except for the lactose, all ingredients were Oxoid brand. Several bile salts were titrated using local water sources as directed by Burman (1955). The optimum bile salt concentration in the Modified MacConkey Medium was found to be 0.3%.

The formulation of the yeastrel medium follows:

Peptone	40 g
Yeast Extract	6 g
Lactose	30 g
H ₂ O	1000 ml

Adjust pH to 7.0, dispense in 20 ml lots in screw cap tubes and autoclave at 117.6 C for 10 minutes.

The differential medium was Modified MacConkey agar:

Peptone	1.0 g
Lactose	3.0 g
Bile Salts	.30 g
Brom-Cresol Purple ¹	3.0 ml
Agar	1.5 g
H ₂ O (pH 7.4)	100 ml

The medium was brought to a rolling boil to dissolve the agar, cooled and poured into sterile petri dishes. Plates were held for not more than 48 hours under refrigeration before use.

3. McCarthy MF Technic

The original formulation of the McCarthy (1961) MF technic was also a two step method. The enrichment medium used was a single strength Lauryl Tryptose Broth. The differential medium was M-Endo containing 25% less water than the standard methods formulation plus 1.5% agar.

The method was modified in this study by replacing the enrichment medium of McCarthy with the same yeastrel medium used in the MacConkey technic. All filters were incubated at 35 C for 2 hours on enrichment medium followed by 20 hours on the differential medium at 35 C.

MF Technic Used for Enumeration of Fecal Coliform Organisms

The m-FC (Rosolic Acid) procedures used for the enumeration of fecal coliform organisms at elevated incubation temperature were those outlined by Geldreich et al (1965). The m-FC Broth and Bacto Rosolic Acid were furnished by Difco Laboratories, Detroit, Michigan. Formulation and preparation of the final medium was made in accordance with recommendations of the manufacturer².

Samples were incubated for 24±2 hours at 44.5±0.2 C in a water bath. Filter and pad were placed inverted in pre-warmed screw cap jars and submerged in the water bath. Warm-up time for the filter and pad in the submerged jars was approximately 2 hours.

¹One percent alcoholic solution. Dye certified by Biological Stain Comm. for use in the preparation of media.

²Difco Technical Information Report dated Sept. 1964. 262.

General Procedure

All samples were collected and transported to the laboratory according to APHA Recommended Procedures for the Bacteriological Examination of Sea Water and Shellfish (Third Edition, 1962).

When necessary, dilutions were made with phosphate buffered dilution water (pH 7.0). Buffered water was also used to rinse MF apparatus between samples. All dilutions of each sample were filtered in duplicate.

After the prescribed incubation period colonies were counted with the aid of a stereoptic microscope at 10X magnification. Colonies from each differential MF medium were enumerated by characteristic color and/or sheen for comparisons with the multitube method. Only those colonies with typical coloration or sheen were considered in calculating the MF results (Table 1).

Table 1. Colonial characteristic on certain membrane filter media

Name of MF Medium	Typical Colonies	Atypical Colonies
M-Endo	Dark red with metallic sheen	Wine, red, colorless
McCarthy	Dark red with metallic sheen	Wine, red, colorless
MacConkey	Yellow	Green, blue, colorless
m-FC Broth	Blue	Green, pink, white

Confirmation of selected colonies of each color was made on each sample. Approximately 50% of the typical colonies were picked to lactose broth. In addition approximately 20% of each of the other colony types were picked to lactose broth.

The inoculated lactose tubes were incubated at 35 C for 24 hours. Those showing gas production in 24 hours were confirmed in Brilliant Green Bile Broth and EC Medium at 44.5 C. Those colonies that failed to produce gas in the lactose broth after 48 hours incubation at 35 C were designated as non-coliform organisms. Unconfirmed colonies in Brilliant Green Bile Broth in 48 hours were designated non-coliform organisms; those that failed to produce gas in EC Medium in 48 hours were designated as non-fecal coliform organisms.

Data Analysis:

Fresh water samples were arbitrarily placed into either the samples with a coliform MPN of less than 1000/100 ml or with more than 1000/100 ml. Only five of the water samples in the less than 1000 range were as high as 790. MPN's of 75% of these samples were 240 or less. MPN's of polluted samples of greater than 1000 ranged from 1300 to 690,000,000. Over 70% of these samples had a MPN of 5000 or more.

Ratios of the various MF counts to multitube MPN's were plotted on probability plots as described by Velz (1951). Plots were made for each MF technic evaluated. For the coliform data each technic was plotted for estuarine waters as well as unpolluted and polluted fresh water. Due to the limited data for the fecal coliform MF technic all samples were placed in one group.

RESULTS

MF Technic for Coliform Organisms:

The estuarine water samples were those collected from the laboratory salt water intake line. The salinity of these samples ranged from 24 o/oo to 31 o/oo. Turbidities were generally at 5 units or less. However, on several occasions during high runoff the turbidity was as high as 20 units.

Results of the 10, 50 and 90 percentile levels and median values for each MF technic are shown in Table 2. The standard M-Endo technic had the lowest ratio to the multitube method of the three MF methods tested. In estuarine samples the 90 percentile level was only 1.20 with a 50 percentile level of 0.27 and a median of 0.30. The MacConkey and McCarthy MF ratios and medians were similar for the estuarine samples. The 50 percentile levels were 0.40 with medians of approximately 0.50. These levels were somewhat higher than those of the M-Endo technic but lower than the desirable level of 1.00.

In fresh water with coliform MPN's of less than 1000/100 ml the standard M-Endo technic and the MacConkey method showed comparable ratios of 0.80 and 0.90 respectively at the 50 percentile level and 6.10 at the 90 percentile level. The M-Endo method median was 0.70 whereas the MacConkey technic median was 1.00. The McCarthy method results were much higher than the multitube MPN's at both the 50 and 90 percentile levels. The extreme range from 0.20 to 82.0 could be attributed in part to the small number of samples examined.

All three technics compared favorably with the multitube method when applied to the water samples with coliform MPN's greater than 1000/100 ml. The McCarthy technic was found to be equivalent to the multitube MPN with a 50 percentile level of 1.00 and a 90 percentile level of 2.40.

Confirmation by colony types for each technic is shown in Table 3. The McCarthy MF technic showed consistently higher percentages of confirmed typical colonies in the three types of samples tested. However, only 78% of the typical colonies were confirmed by this method in the unpolluted water samples. The MacConkey method had the lowest confirmation of typical colonies in all samples except the unpolluted water.

There was a high incidence of confirmation of wine colored colonies in the M-Endo and McCarthy MF technic as well as green colonies from the MacConkey method. Confirmation of other atypical colony types by each method ranged from a high of 36% for red colonies by the McCarthy method to only 9% for colorless colonies by the MacConkey technic. Confirmation of colorless colonies from the other two MF technics was too high to be disregarded.

MF Technic for Fecal Coliform Organisms:

A limited number of samples (46) were tested by the m-FC Broth technic and the multitube method. The results from all environments are shown in Table 4. The range of the m-FC Broth technic as compared to the multitube method was 0.13 to 4.40 with a 50 percentile level of 0.74. The median for these 46 samples was 0.75.

Confirmation of colonies from the m-FC Broth technic is shown in Table 5. Fecal coliforms were confirmed in 88% from typical blue colonies. The green colonies, although few in number, had a higher percentage of confirmation (89.5%). More than 25% of the colorless colonies from this method were confirmed as fecal coliform organisms.

DISCUSSION

Major pitfalls in all MF technics tested were noted. Extreme differences between MF technics and comparative multitube tests were shown in both estuarine and fresh water with coliform MPN's of less than 1000/100 ml. Better correlation between MF technics and multitube tests was shown in fresh water samples with coliform MPN's of greater than 1000/100 ml.

The most promising MF technic in this study was the McCarthy MF. The number of typical counts was consistently higher where enrichment was used. Several attempts were made to find the optimum time for the enrichment incubation. It is suggested that the enrichment incubation period be set at a minimum of two hours.

One factor in the MF/MPN ratios was the failure of coliform organisms to form typical color and/or sheen. The high rate of confirmation of wine colored colonies in the M-Endo and McCarthy technics as well as the green colonies in the MacConkey technic would indicate that many coliform organisms were present which would not be counted by routine methods. Several modifications, including titration of various dyes and salts have not improved the recovery. The failure to observe these coliform organisms is probably one of the major factors responsible for the low MF/MPN ratios of the various MF technics. Further research is indicated to eliminate this difficulty.

The preliminary investigations using m-FC Broth for the enumeration of fecal coliform organisms by the MF technic was encouraging. Placing the filtered sample directly on the differential medium and incubating at the elevated temperature could possibly account for some of the deleterious effects on certain fecal coliform organisms. These factors may account for the exceedingly high rate of confirmation in the green colonies observed. Due to the similarity of the shades of blue and green it was difficult to distinguish between these two colors on many of the filters. Since 89.5% of the green colonies were confirmed perhaps these colonies should be included in the typical colony classification. If fecal coliform organisms are to be considered in the bacteriological examination of shellfish growing areas then efforts should be made to make further evaluations of this and other MF technics for the enumeration of this group.

The main deterrent to the use of MF technics in shellfish growing areas is the lack of selectivity. From the data presented many coliform colonies were grown on the filter but could not be enumerated due to lack of typical color formation. The use of an enrichment incubation period appeared to enhance the enumeration of typical coloration by the McCarthy MF technic.

If the membrane filter technic is to be considered for the enumeration of coliform or fecal coliform organisms in shellfish growing areas further investigations including collaborative studies should be made.

COMMENTS BY PARTICIPANTS

Mr. Kenneth Michener stated that the supplier of medium for the m-FC technic had suggested overnight refrigeration of plates after incubation to give better color differentiation. Mr. Michener also asked about the use of plastic bags in water baths. Mr. Beck replied that refrigeration had not been used as the literature available did not mention this procedure. Mr. Beck commented on pinpoint holes in plastic bags. Mr. Kelly questioned the use of English media on the MacConkey MF technic. Mr. Beck stated that there was no apparent difference except that bile salts had to be titrated against local waters.

REFERENCES

- American Public Health Association. 1962. Recommended procedures for the bacteriological examination of sea water and shellfish. 3rd ed. Am. Pub. Health Assoc., New York. 51 p.
- Burman, N. P. 1955. The standardization and selection of bile salt and peptone for culture media used in bacteriological examination of water. Proc. Soc. Water Treat. Exam. 4:10-25.
- Geldreich, E. C., H. F. Clark, C. B. Huff and L. C. Best. 1965. Fecal coliform-organism medium for the membrane filter technique. J.A.W.W.A. 57(2):208-214.
- Kelly, C. B., W. J. Beck, M. W. Presnell and K. J. Zobel. 1962. Ecological study of bacterial indices of pollution. Shellfish San. Res. Plan. Conf., Purdy, Washington.
- McCarthy, J. A., J. E. Delaney, R. J. Grasso. 1961. Measuring coliforms in water. Water and Sewage Works. 108:238-243.
- Taylor, E. W., N. P. Burman and W. W. Oliver. 1955. Membrane filtration technique applied to the routine bacteriological examination of water. Jour. Inst. Water Eng. 9(3):248-263.
- Velz, C. J. 1951. Graphical approach to statistics: IV Evaluation of bacterial densities. Water and Sewage Works 98:66-73.

Table 2. Ratios of coliform densities from membrane filter technics compared to multitube method

Environment	Samples	Probability Percentile			Median
		10	50	90	
<u>Estuarine Waters</u>					
M-Endo	61	<0.10	0.27	1.20	0.30
MacConkey	60	<0.10	0.40	2.60	0.50
McCarthy	27	<0.10	0.40	2.20	0.45
<u>Coliform MPN less than 1000/100 ml</u>					
M-Endo	36	0.10	0.80	6.10	0.70
MacConkey	54	0.10	0.90	6.10	1.00
McCarthy	15	0.20	3.00	82.00	1.50
<u>Coliform MPN greater than 1000/100 ml</u>					
M-Endo	20	0.20	0.80	3.10	0.80
MacConkey	84	0.20	0.80	3.50	0.90
McCarthy	13	0.40	1.00	2.40	0.90

Table 3. Confirmation of colony types by membrane filter technic as found in various environments

Colony Type	Salt Water				Fresh Water					
	Colonies Picked		Coliform		Colonies Picked		Coliform MPN less than 1000/100 ml		Coliform MPN greater than 1000/100 ml	
			Number	Percent			Number	Percent	Number	Percent
	Picked	Positive	Positive	Positive	Picked	Positive	Positive	Positive	Positive	Positive
<u>M-Endo</u>										
Sheen	1,055	955	90.5		215	145	67.4	1,002	904	90.2
Wine	67	30	44.8		68	20	29.4	124	49	39.5
Red	95	29	30.5		166	27	16.3	213	54	25.4
Colorless	109	25	22.9		106	15	14.2	67	13	19.4
<u>McCarthy</u>										
Sheen	521	493	94.6		141	110	78.0	375	347	92.5
Wine	27	10	37.0		40	12	30.0	35	12	34.3
Red	53	19	35.8		70	21	30.0	47	8	17.0
Colorless	33	8	24.2		81	23	28.4	33	5	15.2
<u>MacConkey</u>										
Yellow	1,485	1,213	81.6		287	197	68.6	982	848	86.4
Green	70	23	32.9		101	22	21.6	107	22	20.6
Blue	122	15	12.3		195	21	10.8	167	21	12.6
Colorless	103	9	8.7		146	15	10.3	115	18	15.7

Table 4. Ratio of m-FC Broth technic for fecal coliform compared with multitube confirmed fecal coliform densities

Environment	Samples	Probability Percentile			Median
		10	50	90	
Fresh and estuarine waters	46	.13	.74	4.40	.75

Table 5. Confirmation of fecal coliform from colony types by m-FC Broth technic in various environments

Colony Type	Salt Water			Fresh Water		
	Colonies Picked	Fecal Coliform Positive	Percent Positive	Colonies Picked	Fecal Coliform Positive	Percent Positive
Blue	153	136	88.8	181	175	96.7
Green	19	17	89.5	27	24	89.0
White	34	9	26.4	80	16	20.0
Pink	31	6	19.4	31	1	3.2

BACTERIOLOGICAL STUDIES ON COMMERCIAL PROCESSING OF OLYMPIA
OYSTERS (OSTREA LURIDA)

G. J. Vasconcelos

INTRODUCTION

Bacteriological changes occurring in Pacific oysters during processing have been studied at this laboratory (Zobel, 1961) (Presnell, 1962). Since Olympia oysters are approximately 1/40 to 1/60 the size of Pacifics the time required to shuck a given volume of Olympia oysters is comparatively greater than that needed for the same volume of Pacific oysters. Depending on number of shuckers and size of plant the interval between shucking and final packing of Olympia oysters may vary by several hours.

This study was undertaken in conjunction with other Olympia oyster storage studies in an attempt to determine the changes that may occur in the density of certain bacterial indices during the commercial processing of this product. Samples were collected from two commercial plants located in the Shelton-Olympia area. A total of twelve lots was examined to include the factors of seasonal variation. Collection of these samples was made possible through the cooperation of the Olympia Oyster Growers Association.

MATERIALS AND METHODS

Collection of Samples:

Samples were collected alternately from two commercial plants during the period November 1964 through August 1965 in order to determine the possible influence of season.

Shellstock samples were collected in plastic bags at the culling house immediately after culling and packed in crushed ice for transportation to the laboratory.

Shucked oysters were taken from three sampling points on the shucking bench. The first, or zero minute sample, was obtained by having the shucker shuck approximately 2 oz. of oyster meats directly into a sterile open-mouth glass container. The second sample was taken immediately after the shucker had completed filling a small 1-2 quart stainless steel pan, as shown in Fig. 1. The observed time required in filling this pan ranged from 45-75 minutes, depending upon the speed of the shucker and the size and quality of the oysters being shucked. The third and final sample was obtained after the shucker, or shuckers, had completed filling a large stainless steel pot. This lapse between the taking of the first and third shucked sample varied from 50 to 300 minutes, depending upon the number of people shucking.

An outline of the various points sampled during processing is given in Table 1. Also included in this table is a range of results encountered at each sampling point.

After completion of the shucking procedure, the large pot of oyster reats was immediately taken to the packing room for washing, rinsing and packing. Following the rinsing process, 2 oz. samples were collected from the skimmer table and packed into sterile open-mouth glass containers (Fig. 2).

All samples including both shucked and shellstock were placed in crushed ice immediately after collection and transported to the laboratory in insulated, refrigerator containers. Maximum time elapsing between collection and actual examination of sample did not exceed six hours.

Examination of Samples:

Upon arrival at the laboratory, samples were examined for the presence of coliform and fecal coliform organisms as well as organisms enumerated by the 35 C Standard Plate Count in accordance with methods described in APHA Recommended Procedures for the Bacteriological Examination of Sea Water and Shellfish (Third Edition, 1962). In addition pH determinations were made from 10 ml homogenized portions of each sample.

RESULTS

A summary of the results obtained from twelve lots is shown in Table 2. Coliform MPN's as well as 35 C plate counts are expressed as geometric means. Because of an excessive number of indeterminately low values, fecal coliform MPN's are expressed in Table 2 as medians. All pH values are expressed as arithmetic means. Although the shucking procedures varied slightly at each plant, results were similar enough to be combined and analyzed as a group. In comparing the influence of season on changes in bacterial content during processing, little difference was noted.

In comparing the results in Table 2, the first significant change in bacterial levels occurred during the period required to fill the small 1-2 quart pan. During the average period of 60 minutes slight increases occurred in coliform and plate count densities while fecal coliform MPN's remained unchanged. The pH during this period did not change.

The most significant numerical change in coliform and plate count densities occurred during the interval required to fill the large stainless steel pot. During this interval the coliform MPN and plate count densities increased fourteenfold and sixfold respectively. In contrast, little change occurred in fecal coliform density during this same interval. The pH values again remained unchanged.

As shown in Table 2, a distinct reduction occurred in plate count density following the washing and rinsing process. However, coliform and fecal coliform MPN's remained practically unchanged. Since it was the practice in both plants to wash the oyster meats immediately after filling the large pot the time interval was not recorded.

Changes in coliform and plate count densities that occurred during filling the large pot were greater in one plant than in the other. This change can, in part, be attributed to the difference in time required at each plant to fill the large pot. One plant, in which only one person was assigned to shucking Olympia oysters required an average time of 257 minutes to complete the shucking process. In the other plant four or five shuckers worked collectively and completed the same process in an average time of 89 minutes.

DISCUSSION

The bacterial index that showed the least change during the entire shucking and washing process was the fecal coliform group, enumerated by the EC test. Coliform and plate counts on the other hand increased progressively during the shucking process and then dropped slightly or remained the same after washing. The pH values remained practically unchanged.

From the results obtained in this study it would appear that the most stable and precise index of sanitary quality of shucked Olympia oysters is the fecal coliform group. The stability of this index has been substantiated by other studies both here (Zobel, 1961) (Presnell, 1962) and on the East Coast (Presnell & Kelly, 1961).

With the exception of one lot in the summer series, season appeared to have little influence on changes in bacterial content during processing. A review of previous studies on commercial processing of Pacific oysters indicated the same lack of seasonal influence (Presnell, 1962).

The prolonged time necessary to proceed through a normal shucking process appeared to be a major contributing factor to the progressive increase observed in coliform MPN's and plate count values. With the exception of fecal coliforms, the pattern followed by the other two index organisms was one of a progressive increase throughout the shucking procedure. Washing the oysters immediately after shucking appears to be a favorable method of reducing bacterial levels extrinsic to the oyster. However, bacterial levels attained by this method were rarely as low as the first or zero minute sample. This would tend to indicate the possibility of some internal multiplication.

Exposure of Olympia oyster meats to room temperature (68-74°F) for extended periods of time, led to definite increases in coliform and plate count densities. Although the bacterial levels encountered in this evaluation generally conformed to established criteria, the possibility exists that increases beyond recommended limits could occur during processing, especially if the initial counts were high. The increase in certain index organisms during the shucking procedure indicates the necessity of either shortening the shucking time to a period comparable with Pacific oysters or lowering the temperature of shucked oyster meats during the interim of shucking and packing.

COMMENTS BY PARTICIPANTS

Mr. Felsing inquired about the ambient room temperature and whether or not temperatures of oysters in containers were taken. Mr. Vasconcelos replied that the room temperature ranged from 68-74°F in the shucking room. No attempt was made to determine the actual temperature of the oysters in the containers.

Mr. Foster asked if two bacteriological criteria should be considered. Mr. Kelly noted that the plate count was related to time and temperature. The use of the plate count would show minor infractions of temperature standards whereas the fecal coliform might be more closely associated with an increase in enteric pathogens.

Mr. Girard indicated that one group of organisms increased when the product was handled for a relatively long period of time. The question was raised as to whether or not a standard of quality at the plant and a separate standard for the finished product should be established. Mr. McMillin wanted to know just how serious this problem would be in the plant. The chilling of oysters immediately after shucking would be good. A long interval between shucking and packaging should be avoided. However, Mr. McMillin would like to have definite criteria to follow.

REFERENCES

- American Public Health Association. 1962. Recommended procedures for the bacteriological examination of sea water and shellfish (3 ed.) Am. Pub. Hlth. Assn., Inc. New York
- Presnell, M. W. 1962. Bacterial changes during processing of Pacific oysters. (Paper presented at Shellfish Sanit. Res. Confer., Purdy, Wash.).
- Presnell, M. W., and C. B. Kelly. 1961. Bacteriological studies of commercial shellfish operations on the Gulf Coast. Sanit. Eng. Center Tech. Rep. F61-9. U. S. Public Health Service.
- Zobel, K. J. 1961. Bacteriological and chemical studies of commercially handled shellfish. (Paper presented at Shellfish Sanit. Res. Confer., Purdy, Wash.).



Fig. 1 Stainless steel pot and pan

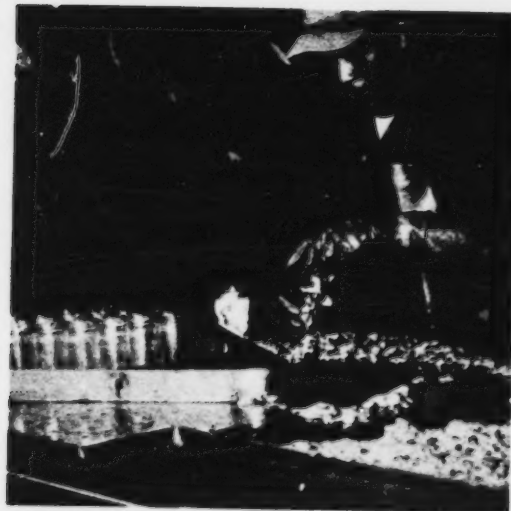


Fig. 2 Collection of samples at skimmer table

Reproduced from
best available copy.



<u>Sampling Point</u>	<u>Range of Results</u>
Shellstock Sample (Taken from Culling House)	Coliform MPN: 20 - 7,000 Fecal Coliform MPN: <18 - 3,900 35 C Plate Count: 1,200 - 14,000
Initial Shucked Sample (Taken at Shucking Bench)	Coliform MPN: 20 - 3,300 Fecal Coliform MPN: <18 - 2,400 35 C Plate Count: 1,300 - 76,000
Sample Taken after Filling 1-2 Quart Pan (Taken at Shucking Bench)	Coliform MPN: 20 - 3,300 Fecal Coliform MPN: <18 - 1,300 35 C Plate Count: 1,100 - 92,000
Sample Taken after Filling Stainless Steel Pot (Taken at Shucking Bench)	Coliform MPN: 130 - 540,000 Fecal Coliform: <18 - 2,400 35 C Plate Count: 4,100 - 340,000
Sample Taken as Commercially Packed (Taken from Skinner Table)	Coliform MPN: 340 - 240,000 Fecal Coliform MPN: <18 - 1,000 35 C Plate Count: 4,000 - 140,000

Table 1. Sampling points and range of results encountered during commercial processing of Olympia oysters

Table 2. Changes in bacterial densities and pH of commercially processed Olympia oysters (12 lots)

Sample	Time in min. ^b	Coliform MPN/100g ^b	Fecal Coliform MPN/100g ^a	35 C Plate Count/g ^b	pH
Shellstock		250	20	6,200	6.6
Initial shucked sample	0	330	20	7,500	6.4
After filling 1-2 qt. pan	60	450	20	11,000	6.4
After filling 3 gal. pot	140	4,800	45	45,000	6.4
Commercial pack		4,800	40	16,000	6.3

^aMedian values (12 lots)

^bGeometric means (12 lots)

BACTERIOLOGICAL STUDIES ON STORAGE OF SHUCKED
OLYMPIA OYSTERS (OSTREA LURIDA)

T. H. Ericksen

INTRODUCTION

Previous studies have shown the effect of storage time and temperature on the sanitary quality of shucked Eastern oysters (Presnell and Kelly, 1961; Kelly, 1964) and shucked Pacific oysters (Presnell, 1962; Hoff, Beck, and Presnell, 1964; Kelly, 1964). Indices used in these studies included coliform and fecal coliform MPN, 35 C plate count and pH. The change in the temperature requirement for refrigeration of shucked shellfish adopted by the 5th National Shellfish Sanitation Workshop (1964) was based primarily on the information supplied by these studies.

Production and marketing of Olympia oysters is increasing. Since information on the effects of storage on shucked Olympia oysters was limited, it was suggested at the 1964 Northwest Shellfish Sanitation Research Planning Conference that such studies be undertaken. This study was initiated to investigate the effects of storage time and temperature on the bacteriological quality of shucked Olympia oysters as determined by the coliform and fecal coliform MPN, 35 C plate count and pH. Determination of possible differential growth patterns in the fecal coliform group of organisms was also studied by IMViC tests on positive EC tubes at selected sampling intervals. The study consisted of 12 lots of oysters and was initiated in November, 1964 and continued through August, 1965.

MATERIALS AND METHODS

Storage of Oysters:

The fresh commercially packed Olympia oysters were obtained alternately from two different sites through the cooperation of the Washington State Department of Health and the Olympia Oyster Growers Association. The oysters were processed in the usual manner and placed in sterile 4 oz. screw-cap jars (2 oz. per jar). The jars were immediately placed in crushed ice for transport to the laboratory. Upon arrival at the laboratory the jars were divided into groups and stored at designated temperatures.

Storage facilities consisted of a chest freezer for -20 C storage, a refrigerator case containing crushed ice for iced storage, and modified refrigerators equipped with special thermoregulators and air circulators for the 3 C and 10 C storage. The chest freezer and refrigerators were also equipped with constant recording temperature devices.

Sampling:

A sample consisted of the oyster contents from one of the 4 oz. jars. Two samples were examined immediately upon arrival at the laboratory and provided the 0 hour sample for all four storage temperatures. Subsequent samples were examined according to the following schedule:

1. -20 C - 0 hour sample, 1, 3, 6, 9 and 12 months
2. Iced - 0 hour sample, 1, 2, 4, 7, 10, 15 and 20 days
3. 3 C - 0 hour sample, 1, 2, 4, 7, 10, 15 and 20 days
4. 10 C - 0 hour sample, 1/2, 1, 1-1/2, 2, 3, 4, 5, 6 and 7 days.

Bacteriological Examination:

Each sample was blended in a sterile blender jar for 15 seconds. A 10 ml portion was removed for electrometric pH determination. Phosphate buffer equal in weight to the remaining shellfish meats and liquor was added to the blender jar and blending was continued for an additional 75 seconds. Bacteriological examinations included coliform and fecal coliform MPN and 35 C plate count as outlined in APHA Recommended Procedures for the Bacteriological Examination of Sea Water and Shellfish (1962). At selected intervals IMViC tests were carried out on all positive EC tubes.

RESULTS

The -20 C storage phase of the study has not been completed. Data available to date from the -20 C storage indicate slight decreases in the coliform MPN, fecal coliform MPN and 35 C plate count as storage progressed. The pH remained relatively the same. These data will be reported upon completion of this phase of the study.

Geometric means for the coliform MPN's and 35 C plate counts for the fall, winter, spring and summer lots of oysters stored in ice, 3 C and 10 C were compared. No seasonal variations were apparent. Therefore, all 12 lots were grouped together and the geometric means were calculated at each of the three temperatures as storage progressed.

The coliform data are shown in Fig. 1. The coliform MPN of oysters stored in ice showed a slight decrease for the first two days, followed by a stable period to the tenth day. This was followed by a gradual increase through the twentieth day of storage. The coliform MPN for oysters stored at 3 C exhibited a one day decrease followed by a regular increase throughout the twenty days of storage. The coliform MPN for the 10 C storage showed a steady increase throughout the seven day storage period.

The 35 C plate count data are shown in Fig. 2. The plate count for iced storage showed an initial two day lag period followed by a gradual increase through the twenty day storage period. The 3 C storage indicated a gradual increase to the seventh day followed by a steady increase for the remainder of storage. The plate count for the 10 C storage showed a slight decrease for the first day followed by a uniform increase as storage progressed.

Median values were used to analyze the fecal coliform MPN data because of the presence of indeterminately low values (<18). Median values for the fecal coliform MPN's for oysters stored in ice and at 3 C for the fall, winter, spring and summer lots were compared. No seasonal variations were apparent. Therefore, all 12 lots were grouped together and the medians were determined at each of the two temperatures as storage progressed. The fecal coliform data for oysters stored in ice and 3 C are shown in Fig. 3. The fecal coliform MPN's for oysters stored in ice and 3 C showed little change throughout storage.

Comparison of fecal coliform changes which occurred at 10 C revealed no seasonal influence. However, it was observed in three lots, one each in the winter, spring and summer, that the fecal coliform MPN increased during storage at 10 C. Fecal coliform MPN's of the remaining nine lots remained stable. Therefore, the fecal coliform MPN data for the three lots were combined and the geometric means calculated. Due to indeterminately low values (<18) medians were determined on the remaining nine lots. A comparison of the results for the fecal coliform MPN's of oysters stored at 10 C are given in Fig. 4. The nine lots indicated little change in fecal coliform MPN as storage progressed. The fecal coliform MPN data for the three lots showed an increase of almost a hundredfold by the fifth day of storage, followed by a slight decrease through the seventh day of storage.

The pH data are shown in Table 1. The pH values for the twelve lots were similar. Therefore, the values were grouped together and the arithmetic means determined for each temperature as storage progressed. These data indicated a gradual decrease as storage progressed at the three temperatures. The decrease ranged from 6.4-5.8 in ice; 6.4-5.6 at 3 C and 6.4-5.4 at 10 C.

Results of the IMViC tests at selected intervals on all positive EC tubes are given in Table 2. These results showed 97 percent of the isolates were E. coli and 13 percent were A. aerogenes.

DISCUSSION

This study indicated that the coliform MPN and 35 C plate count of shucked Olympia oysters increased as storage progressed. Therefore, these two indices seemed to be influenced by duration and condition of storage. In contrast, fecal coliform levels in all lots stored in ice and at 3 C (37.4 F), and in nine of the lots stored at 10 C (50 F) remained relatively stable. In three lots stored at 10 C (50 F), fecal coliform MPN's increased. Since these three lots were collected at different times in the year, this did not seem to be a seasonal influence. It was observed that the initial fecal coliform levels in these lots were about tenfold higher than those of the other nine lots. Therefore, it appears that the initial concentration may have influenced the pattern of change of fecal coliform MPN in oysters stored at 10 C (50 F).

The fecal coliform MPN as enumerated by the EC test seemed to be influenced least by duration or condition of storage. Previous storage studies have indicated this relationship also applies to shucked Pacific oysters (Presnell, 1962; Hoff, Beck and Presnell, 1964; Kelly, 1964) and shucked Eastern oysters (Kelly, 1964). An increase in fecal coliform MPN at 10 C (50 F) storage was also observed in shucked Pacific oysters (Hoff, Beck, and Presnell, 1964). These data also lend supporting evidence for the shucked shellfish storage temperature range adopted as a recommendation by the 5th National Shellfish Sanitation Workshop (1964).

Although large increases in bacterial population occurred as indicated by the coliform MPN and 35 C plate count at the three temperatures, the pH exhibited little change in ice and 3 C (37.4 F) and the greatest change at 10 C (50 F). Because of the slight changes in pH of oysters stored in ice and 3 C (37.4 F), the pH as an indicator of the sanitary quality of shucked Olympia oysters was of limited value.

The IMViC data from selected positive EC tubes indicated no differential growth patterns or seasonal variations in fecal coliform content of the oysters. The predominant fecal coliform present was E. coli. The number of A. aerogenes found was almost all from a single lot of oysters. This lot was not one in which an increase in the fecal coliform MPN occurred. A possible explanation of this finding may be the fact that in the two weeks prior to collection of these oysters a rainfall of 6.6 inches was experienced. This would increase the runoff from the surrounding land areas into the overlying waters. Therefore, the numbers of these bacteria in the overlying waters could have been increased greatly.

In conclusion the data obtained from this study support the findings of similar shellfish storage studies. The storage temperature of choice for shucked Olympia oysters was the iced storage. Storage at the lower temperatures would tend to prolong the shelflife of the product as determined by the indices of sanitary quality employed.

COMMENTS BY PARTICIPANTS

Mr. Max Hays inquired as to the volume of the storage container. Mr. Ericksen replied that a 4 oz. screw cap jar was used.

Mr. John Girard asked if any determinations were made on the time temperature of oysters stored in ice. Mr. Ericksen replied that none had been attempted.

Mr. C. B. Kelly made the general observations that another species of oyster was behaving the same as the Pacific and Eastern oyster during storage. The Olympia oyster should be placed in the same criteria used for other species.

REFERENCES

- American Public Health Association. 1962. APHA Recommended Procedures for the Bacteriological Examination of Sea Water and Shellfish. 3rd edition. Am. Pub. Hlth. Assn., Inc., New York.
- Hoff, J. C., W. J. Beck and M. W. Presnell. 1964. A study of the applicability of several indices as sanitary quality indicators in commercially packed Pacific oysters (Crassostrea gigas). Proc. N.W. Shell. sanit. Res. Plann. Confer. PHS Pub. No. 999-FP-4.
- Kelly, C. B. 1964. Time-temperature effect on bacteriological quality of stored oysters, p. 193-202. In Proceedings 5th National Shellfish Sanitation Workshop. U. S. Dept. Hlth., Educ., and Welfare, Washington, D. C.
- Presnell, M. W. 1962. Studies on stored Pacific oysters. (Paper presented at Shellfish Sanit. Res. Conf., Purdy, Wash.).
- Presnell, M. W. and C. B. Kelly. 1961. Bacteriological studies of commercial shellfish operations on the Gulf Coast. Robert A. Taft Sanitary Engineering Center, Technical Report No. F61-9. U. S. Public Health Service.
- U. S. Department of Health, Education and Welfare. 1964. Proceedings 5th National Shellfish Sanitation Workshop. Washington, D. C. 46p.

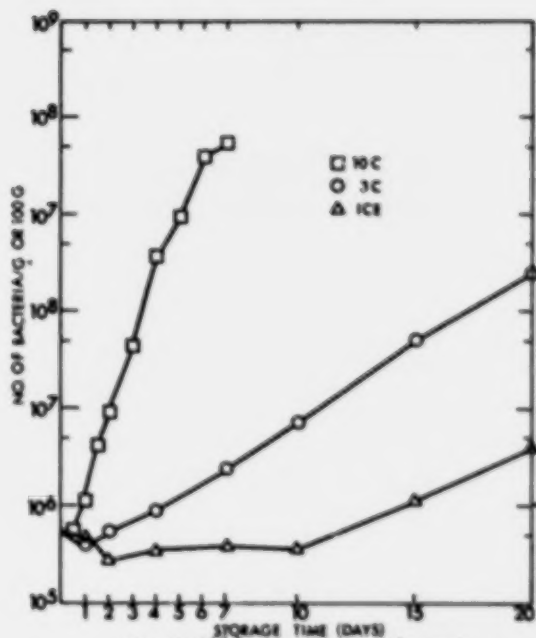


FIG. 1 CHANGES IN COLIFORM MPN OF SHUCKED OLYMPIA OYSTERS STORED IN ICE AT 3°C AND 10°C (12 LOTS)

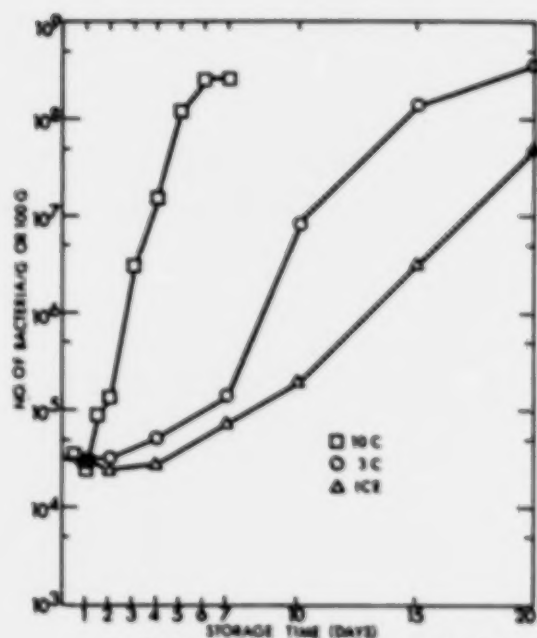


FIG. 2 CHANGES IN 25°C PLATE COUNT OF SHUCKED OLYMPIA OYSTERS STORED IN ICE AT 3°C AND 10°C (12 LOTS)

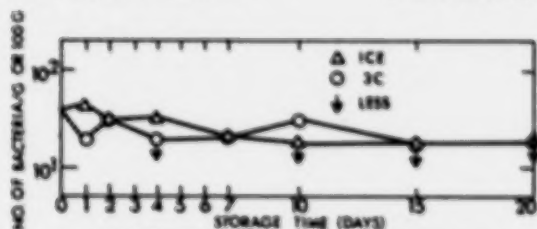


FIG. 3 CHANGES IN FECAL COLIFORM MPN OF SHUCKED OLYMPIA OYSTERS STORED IN ICE AND 3°C (12 LOTS)

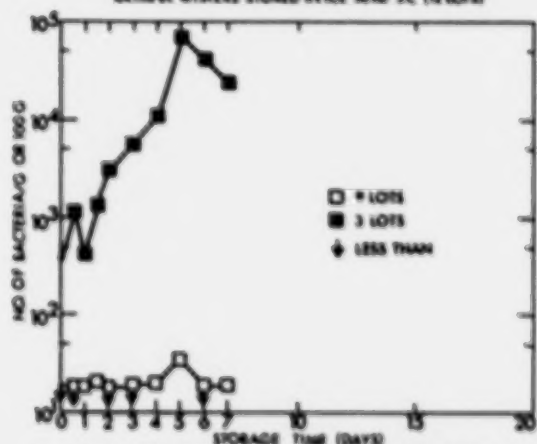


FIG. 4 CHANGES IN FECAL COLIFORM MPN OF SHUCKED OLYMPIA OYSTERS STORED AT 10°C

Table 1. Changes in pH of shucked Olympia oysters stored in Ice, 3 C and 10 C (12 lots)

Storage time (days)	Storage Temperature		
	Ice	3 C	10 C
0	6.4	6.4	6.4
1/2			6.2
1	6.3	6.2	6.1
1-1/2			6.0
2	6.2	6.1	6.0
3			5.9
4	6.1	6.0	5.8
5			5.8
6			5.6
7	6.0	5.9	5.4
10	6.0	5.8	
15	5.9	5.7	
20	5.8	5.6	

Table 2. Coliform type isolated from positive EC tubes at selected intervals during dry storage of shucked Olympia oysters in ice, 3 C and 10 C (12 lots)

Storage	Temperature	E. coli		A. aerogenes		Intermediate	
		No.	Percent	No.	Percent	No.	Percent
0		58	87	9	13		
1/2	10 C	11	100				
1	Iced	30	72	12	28		
	3 C	12	80	3	20		
	10 C	24	80	6	20		
1-1/2	10 C	20	100				
2	Iced	20	100				
	3 C	15	100				
	10 C	39	91	4	9		
3	10 C	17	94			1	6
4	Iced	20	87	3	13		
	3 C	9	69	4	31		
	10 C	58	81	7	19		
5	10 C	62	100				
6	10 C	17	100				
7	Iced	1	14	6	86		
	3 C	1	25				
	10 C	25	100				
10	Iced	12	61	7	39		
	3 C			2	100		
15	Iced	21	96	1	4		
	3 C						
	10 C						
20	Iced	13	88	2	12		
	3 C	5	100				
	10 C						

STORAGE STUDIES ON OLYMPIA OYSTER SHELLSTOCK (OSTREA LURIDA)

George J. Vasconcelos

INTRODUCTION

At the 1964 Shellfish Planning Conference a study of the behavior of several bacterial indices in Olympia oyster shellstock during periods of dry storage and shipment was suggested. Commercially, Olympia oyster shellstock is stored and shipped in much the same manner as Pacific oyster shellstock. Information gathered from industry has indicated that both Pacific and Olympia oyster shellstock are usually shipped and stored at ambient temperatures.

This study was undertaken to determine the relative changes occurring in coliform MPN, fecal coliform MPN and 35 C Standard Plate Count at temperatures encompassing those encountered in commercial operations. Results are based on twelve lots collected and examined during the fall, Winter, Spring and Summer months of 1964-65.

MATERIALS AND METHODS

Collection of Oysters:

The Olympia oysters used in this study were collected alternately from two commercial growing areas in the State of Washington. Each lot was collected after culling and consisted of approximately 620 mature oysters. Prior to collection the oysters had been washed once in fresh water by personnel at the processing plant. All shellstock samples were placed in plastic bags and transported to the laboratory packed in crushed ice. Maximum time elapsing between collection and actual examination of samples did not exceed six hours.

Storage:

Upon arrival at the laboratory each lot was washed for a second time in fresh water and divided into three random groups of approximately 30 each for storage at 10 C, 20 C and 27.5 C respectively.

Oysters were placed in perforated plastic bags and stored in modified refrigerating or incubating units capable of maintaining constant temperatures. Each unit was equipped with a temperature recording device, relay, air circulator and thermostat capable of temperature control to ± 0.5 C.

Sampling:

A sample at each interval consisted of a minimum of twenty tightly closed oysters. In addition "gapers" were examined if more than ten appeared during the interval between sampling time. "Gapers" not used for testing purposes were counted and then discarded at the end of each sampling period.

Sampling times for each temperature were as follows:

1. 10 C - 0 hr., 1, 2, 4, 7, 10, 15 and 20 days.
2. 20 C - 0 hr., 1/2, 1, 1-1/2, 2, 3, 4, 5, 6 and 7 days.
3. 27.5 C - 0 hr., 1/2, 1, 1-1/2, 2, 3, 4, 5, 6 and 7 days.

In each case the 0 hr. sample referred to the first sample tested immediately upon arrival at the laboratory. Duplicate tests were performed on all 0 hr. samples.

Bacteriological analysis:

Each sample of twenty mature, non-gaping oysters was shucked in the prescribed manner and placed in a sterile beaker. The sample was then weighed and poured into a sterile blending jar. After 15 seconds blending time a 10 ml portion of each homogenate was removed and used in the pH determination. Following the addition of an equal weight of phosphate buffered water (pH 7.0), the remainder of the homogenate was blended for an additional 75 seconds. After blending, each homogenate was examined for the presence of coliform and fecal coliform organisms as well as organisms enumerated by the 35 C Standard Plate Count in accordance with APHA Recommended Procedures for the Bacteriological Examination of Sea Water and Shellfish (Third Edition, 1962).

At selected intervals during the course of study EMB isolates from positive EC tubes were subjected to the DNViC test for coliform typing. These tests were employed to determine whether or not differential growth patterns occurred in the oyster during periods of dry storage.

RESULTS

The data presented in this report are based on twelve lots collected and examined during the months of November, 1964 through August, 1965.

Coliform MPN's and 35 C plate counts are graphically expressed in terms of geometric means in Fig. 1 through 3. Because of an excessive number of indeterminately low values, fecal coliform MPN's are expressed in Fig. 1 through 3 as medians.

Season appeared to have little influence on changes in coliform and plate count densities during storage. However, changes in fecal coliform MPN's at 27.5 C during the spring series were somewhat greater than those observed during the fall, winter and summer series.

The results of oyster storage at 10 C for twenty days are shown in Fig. 1. Coliform and fecal coliform MPN's remained practically unchanged throughout the storage period while the plate counts increased about six-fold.

Oysters stored at 20 C (Fig. 2) exhibited an increase in coliform density during the first two days followed by a leveling-off period and then a slight decrease at the termination of the storage period. Similarly the plate counts increased rather rapidly initially followed by a more gradual increase on the third day. In contrast, the fecal coliform MPN's remained practically unchanged.

At 27.5 C (Fig. 3) the coliform MPN's increased initially followed by a steady decline on the third day. In like manner, the 35 C plate counts increased steadily during the first four days, followed by a gradual decrease on the fourth day. Fecal coliform MPN's fluctuated greatly during the first two days, followed by a steady decline during the remaining five-day period.

Changes in pH obtained at the three storage temperatures are shown in Table 1. Shellstock stored at 10 C and 20 C demonstrated a slight but uniform increase in pH ranging from an initial value of 6.6 to a final pH of 6.9 at both temperatures. Changes in pH of shellstock stored at 27.5 C fluctuated greatly, but showed the same general pattern.

Ratios of coliform, fecal coliform and plate count densities of open oysters ("Gapers") to closed oysters are depicted in Table 2. The ratios for coliform and fecal coliform MPN's at 27.5 C ranged from 0.32 to 48.0 and 0.09 to 42,000 respectively. Plate count ratios ranged from a low of 8.4 on the fourth day to a high of 43.8 on the sixth day. On the fifth day extremely high ratios were encountered in all three indices at 27.5 C. "Gapers" at 10 C and 20 C were not always found in sufficient numbers to be examined. However, at 27.5 C (Table 3) a large number of "gapers" appeared after only three-four days of storage.

Olympia oyster shellstock stored at 27.5 C survived for a longer period of time than did Pacific oyster shellstock stored at the same temperature. Survival periods at 10 C and 20 C for both Pacific and Olympia oyster shellstock were the same.

Data from the IMViC testing of positive EC tubes are presented in Table 4. Positive EC tubes, selected at each storage day interval, were examined by the IMViC method and found to contain 100 percent Escherichia coli. As storage progressed, no change in percent E. coli occurred. Consequently no differential growth patterns were observed.

DISCUSSION

Results of this study were similar to those obtained by Hoff, Beck and Ericksen (1964) on Pacific oyster shellstock. Storage at temperatures of 20 C (68 F) or above for extended periods of time appeared to have an adverse effect on the bacteriological quality of Olympia oyster shellstock. At these temperatures, rapid increases occurred in coliform and plate count densities while fecal coliforms remained relatively unchanged. Organisms enumerated by the 35 C Standard Plate Count demonstrated the most uniform response to time and temperature condition of storage. Coliform MPN changes were less uniform. Fecal coliform MPN's at 10 C (50 F) and 20 C (68 F) were least influenced by time-temperature condition, demonstrating once more its stability as an indicator of bacteriological sanitary quality.

The number of "gapers" appearing at 10 C (50 F) and 20 C (68 F) was nominal, indicating that the lower temperatures tend to prolong the storage life of Olympia oyster shellstock. At 27.5 C (81.5 F) large numbers of "gapers" appeared after four to six days of storage. The largest number appeared during the summer months, indicating that during the spawning season the Olympia may be more susceptible to gaping when stored at higher temperatures.

Although the pH increased gradually at 10 C (50 F) and 20 C (68 F) as storage progressed, the change was slight, varying by only 0.3 of a pH unit. In Pacific oyster shellstock the pH indicated little change at these two temperatures (Hoff, Beck, Ericksen, 1964). Possibly acid products produced during dry storage were controlled in some manner by the living oyster. Médcof (1958) reported that acid products produced during dry storage caused erosion (etching) of the glossy inner shell of some quahogs. Erosion of the inner calcareous shell may have in turn neutralized these acid products, thus causing a slight increase in pH.

It would appear from the data presented in this report the storage of Olympia oyster shellstock at temperatures approaching 27.5 C (81.5 F) or above should be avoided. Storage at these temperatures for extended periods of time resulted in rapid increase in organisms enumerated by the 35 C Standard Plate Count as well as increased oyster mortality. As the number of "gapers" increased there was also a subsequent increase in coliform, fecal coliform and plate count ratios (Table 2). Storage at 10 C (50 F) appears to be the most effective and practical means of controlling bacterial multiplication as well as prolonging storage life of Olympia oyster shellstock.

COMMENTS BY PARTICIPANTS

Mr. David McMillin inquired about determination of mortality at 10 C other than by observation of "gapers" and at what day did the greatest number of "gapers" occur at this temperature. Mr. Vasconcelos explained that "gapers" was the only criteria of mortality used in this study. The greatest number of "gapers" at 10 C storage occurred between the 15th and 20th days of storage.

Mr. McMillin was interested in the pH values returning to normal values on prolonged storage. Mr. Vasconcelos thought that this phenomenon may be due to production of acid products reacting with the shell. However, no evidence of shell etching was noted. Mr. Beck explained the limited amount of antimicrobial studies performed but no signs of inhibition of growth of microorganisms tested were noted. Mr. Kelly suggested that rather than only commensal organisms other physiological reasons may be involved.

REFERENCES

- American Public Health Association. 1962. Recommended procedures for the bacteriological examination of sea water and shellfish (Third Ed.) Am. Pub. Hlth. Assn., Inc., New York
- Ericksen, T. H., J. C. Hoff and W. J. Beck. 1964. Storage studies on Manila clams (Tapes japonica) and Native Littleneck clams (Protothaca staminea) shellstock. Proc. N.W. Shell. Sanit. Res. Plann. Confer. PHS Pub. No. 999-FP-4.
- Hoff, J. C., W. J. Beck, and T. H. Ericksen. 1964. Bacteriological study of stored Pacific oyster shellstock. Proc. N.W. Shell. Sanit. Res. Plann. Confer. PHS Pub. No. 999-FP-4.
- Medcof, J. C., 1958. Studies on stored oysters (Crassostrea virginica). Proc. National Shellfish Assoc. 49:13-28.

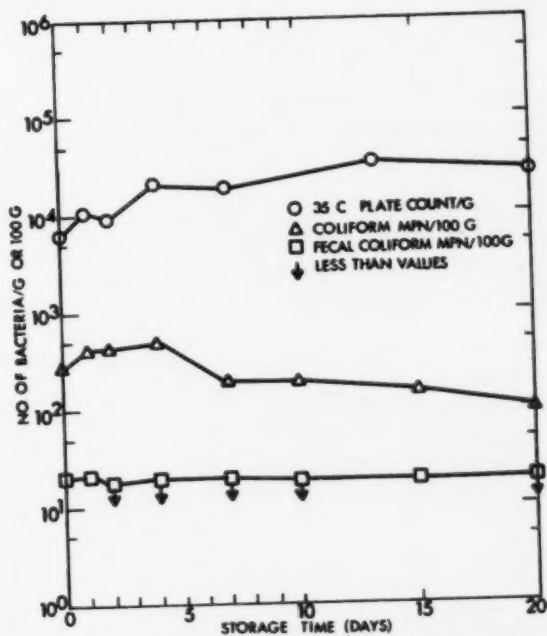


FIG. 1 CHANGES IN COLIFORM MPN FECAL COLIFORM MPN, AND 35 C PLATE COUNT IN OLYMPIA OYSTER SHELLSTOCK STORED AT 10°C (12 LOTS)

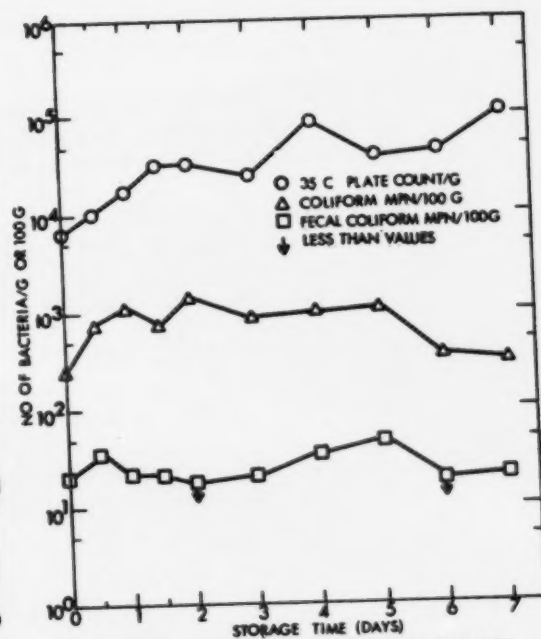


FIG. 2 CHANGES IN COLIFORM MPN FECAL COLIFORM MPN, AND 35 C PLATE COUNT IN OLYMPIA OYSTER SHELLSTOCK STORED AT 20°C (12 LOTS)

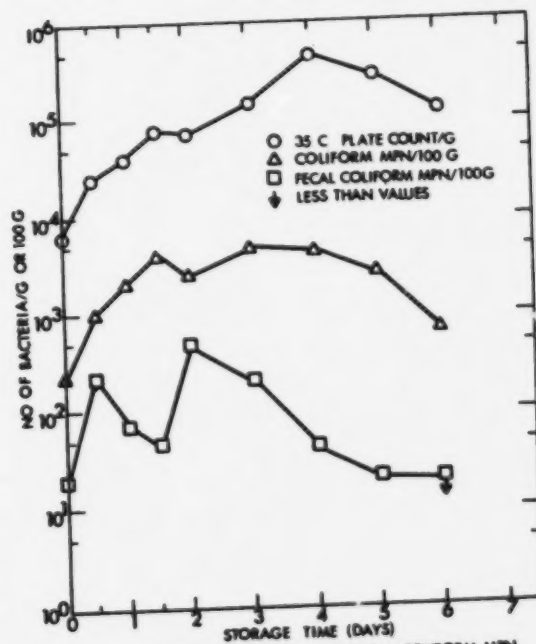


FIG. 3 CHANGES IN COLIFORM MPN FECAL COLIFORM MPN AND 35 C PLATE COUNT IN OLYMPIA OYSTER SHELLSTOCK STORED AT 27.5°C (12 LOTS)

Table 1. Effect of storage time and temperature on
pH of Olympia oyster shellstock

Storage Time (days)	pH ^a of shellstock stored at:		
	10 C	20 C	27.5 C
0	6.6	6.6	6.6
1/2		6.6	6.8
1	6.6	6.7	6.7
1-1/2		6.7	6.8
2	6.7	6.8	6.8
3		6.8	6.9
4	6.8	6.8	6.5
5		6.9	6.8
6		6.9	6.7
7	6.8	6.9	
10	6.8		
15	6.7		
20	6.9		

^aArithmetic mean 12 lots.

Table 2. Comparison of bacterial population in gaping Olympia oyster with closed Olympia oyster (12 lots)

Storage time (days)	Ratio: Bacteria in "Gapers" Bacteria in closed oyster					
	27.5 C			20 C		
	Coliform	Fecal ^a	35 C	Coliform	Fecal ^a	35 C
	MPN	Coliform MPN	Plate Count	MPN	Coliform MPN	Plate Count
1/2						
1-1/2						
2						
3	6.3	0.09	32.0			
4	3.6	3.2	8.4			
5	48.0	42,000	33.3			
6	0.32	1.0	43.8	0.18	2.1	1.1
7						
10						
15						
20						

^aMedian values

Table 3. Average number of "gapers" counted during storage at 10 C, 20 C and 27.5 C

Storage time (days)	Average number of "gapers" appearing at:		
	10 C	20 C	27.5 C
1/2		0	1
1	0	0	1
1-1/2		1	2
2	0	1	2
3		3	12
4	1	4	21
5		4	19
6		3	19
7	2	4	18
10	2		
15	4		
20	5		

Table 4. IMViC analysis of EC positive cultures isolated during storage of Olympia oyster shellstock at 10 C, 20 C and 27.5 C

Storage time (days)	No. Isolates	% E. coli	No. Isolates	% E. coli	No. Isolates	% E. coli
0	58	100	58	100	58	100
1/2	-		15	100	16	100
1	28	100	53	100	22	100
1-1/2	-		12	100	18	100
2	14	100	59	100	19	100
3	-		13	100	17	100
4	42	100	51	100	24	100
5	-		13	100		
6	-		32	100		
7	2	100				
10	30	100				
15	20	100				
20	9	100				

ANTIMICROBIAL AGENTS IN WEST COAST SHELLFISH

W. Jakubowski

INTRODUCTION

Several investigators have demonstrated the presence of antibacterial activity in certain species of shellfish (Li, 1960a, 1960b; Prescott and Li, 1960). The initial isolation of antimicrobial agents was performed using abalone (*Haliotis refescens*). These agents have since been detected in oysters and clams. Crab, flounder, shrimp, lobster and butterfish have also been tested but results have been negative (Li, 1960b).

In some instances antiviral activity and tumor inhibiting substances have been found (Li, Prescott and Jahnes, 1962; Li et al, 1962; Schmeer, 1964). The extracts obtained from a single lot of shellfish have been separated into fractions exhibiting antibacterial activity and other fractions demonstrating antiviral activity. These substances may have significance in maintaining the biological equilibrium of the marine environment. In addition there remains the possibility of clinical application.

With the exception of abalone (*Haliotis refescens*) all extraction and testing had previously been done on East Coast shellfish. In the present study extracts of some bivalves common to Washington, Oregon and California have been tested against standard cultures as a preliminary screening procedure to determine the presence of antibacterial activity. Extracts have been retained frozen for subsequent testing against other bacteria and viruses.

METHODS AND MATERIALS

Sampling:

Shellfish species collected to date and their location by state are shown in Table 1. The collection areas in each state are shown in Table 2. Each lot of shellfish consisted of about 5-15 animals of a single species. Samples were stored in ice for transportation to the laboratory and were placed in a freezer at -20 C until extracts could be prepared.

Preparation of Extracts:

Initially, five different extracts of a single lot of shellfish were prepared in order to determine if one extraction method might be superior to another. The preparation of extracts from one lot of shellfish is shown in Fig. 1. A control, consisting of phosphate buffer only, was prepared at the same time.

The buffer used in preparing extracts consisted of monobasic potassium phosphate solution adjusted to pH 6.9. Shellfish meats were blended with an equal weight of this buffer for 2 minutes in an Oster blender. Extracts were heated to 90-95 C for 30-45 minutes and were centrifuged at 10,000 RPM for 30 minutes at 10 C. The supernatant was passed through a type AA 0.8 micron Millipore filter.

Since antimicrobial activity was not readily detectable in the extracts prepared by the above procedures a revised method of extraction was used on later lots. In the revised method the liquor and meats of a single lot of shellfish were separated. The liquor was heated to 70 C for 30 minutes and then centrifuged at 2100 RPM for 15 minutes. After blending the meats for 60 seconds, phosphate buffer equivalent to one-half the weight of the meats was added. This mixture was then heated to 90-95 C for 30 minutes and centrifuged at 2100 RPM for 15 minutes. Extracts were retained frozen at -20 C until they could be tested.

Testing Methods:

Initial testing of extracts for antibacterial activity was performed using an absorbent paper disc method. However, because of inconsistent results reported in the literature (Li, 1960a) with this method several different methods were employed.

Extracts were tested against a penicillin-sensitive strain of Staphylococcus aureus (FDA 209), a coagulase-positive staphylococcus and a beta-hemolytic streptococcus. Extracts were first tested for sterility by plating duplicate 1 ml portions using Trypticase Soy Agar. Plate counts were done after incubating at 35 C for 48 hours.

1. Disc Test

A 24 hour culture of the test organism grown at 35 C in Brain Heart Infusion Broth was incorporated into Trypticase Soy Agar. A sufficient amount of the 24 hour culture was used to produce confluent growth. Absorbent paper discs 7 mm in diameter were immersed in the extract being tested. After placing the discs on the solidified agar surface, plates were incubated at 35 C for 24 hours and examined for evidence of a zone of inhibition.

In a variation of this method a 10⁻⁴ dilution of the test organism was spread on the surface of agar plates. Paper discs immersed in the extract were placed on the agar surface and plates were incubated at 35 C for 24 hours.

2. Tube Dilution Test

The tube dilution method according to Li (1960a) employs serial dilutions of a 24 hour culture of the test organism. Tenfold dilutions of a 24 hour culture were made in phosphate buffer solution using a 10^{-4} dilution as the highest concentration. A 13 mm x 100 mm test tube containing 1.8 ml of Brain Heart Infusion Broth and 0.2 ml of extract was inoculated with 0.1 ml of each dilution. After 18 hours incubation at 35 C 0.1 ml from each tube was spread on the surface of previously dried Trypticase Soy Agar plates. Plate counts were performed after incubating at 35 C for 24 hours.

3. Agar Well Test

Using a sterile glass tube 7 mm in diameter wells were punched into the surface of Trypticase Soy Agar plates previously spread with various dilutions of the test organism. About 0.1 ml of extract was placed in each well and plates were incubated overnight at 35 C. They were then examined for evidence of inhibition.

4. Bacteriophage Activity Test

Extracts were tested for activity against an Escherichia coli C₂ bacteriophage. A mixture of 0.5 ml of extract and 0.5 ml of a phage suspension containing about 240 PFU/ml was incubated for 5 minutes at 45 C. Then 1 ml of semi-solid agar and 0.1 ml of the phage specific E. coli C₂ culture were added. This mixture was spread on agar plates and plaque counts were done after 48 hours incubation at 28 C.

RESULTS

To date approximately ninety extracts representing three species of oysters and six species of clams from various locations in Washington and Oregon have been tested for antimicrobial activity. No samples have been obtained from California. Samples extracted thus far have been obtained during winter and early spring. Only eight samples have been tested by the tube dilution method (Table 3).

No evidence of inhibition of growth by the disc method was observed with extracts obtained from nine oyster samples. In many cases enhancement of growth was noted with all three test organisms.

Of the clam samples tested only an extract obtained from the Cockle clam (Clinocardium nuttalli) indicated very slight inhibition of the two staphylococcus cultures by the disc and tube dilution methods. A 1 mm zone of inhibition was produced against the S. aureus (FDA 209) culture. Growth of the coagulase-positive staphylococcus was inhibited in a 10^{-7} dilution of the organism. As was observed with the oyster extracts many of the clam extracts produced enhancement of growth.

Extracts were also prepared from whole, live abalone (Haliotis kamschatkana) which had been kept in an aquarium for one year. An extract of the meat and juice obtained from the meat indicated slight inhibition when tested against S. aureus (FDA 209) by the tube dilution method. No inhibition was demonstrated by these extracts when using any of the other methods.

Results of the tests for activity against an E. coli C₂ bacteriophage shown in Table 4 indicate that the phage was protected by the extracts. After 5 minutes incubation at 45 C plaque counts of preparations containing the extracts were 3 to 5 times higher than a control preparation in 0.1% peptone water.

DISCUSSION

As reported in the literature the absorbent paper disc method has given inconsistent results (Li, 1960a, 1960b). This is believed to be due to the non-diffusability of the antimicrobial agent in a solid medium. For this reason a tube dilution method was employed. Li et al (1962), determined the antimicrobial agents to be protein in nature with a molecular weight of 5,000 to 10,000. They are nondialyzable, thermostable and are not digested by pepsin.

A dilution of the organism rather than a dilution of the extract was used because of the presumably low concentrations of the antimicrobial agent. However, this method may be unreliable if very dilute suspensions of the test organisms must be used. This may be overcome by concentrating the extracts using freeze-drying procedures. On the other hand, in his initial work with crude abalone and oyster extracts, Li, (1960a, 1960b) was able to demonstrate inhibition of S. aureus, FDA 209 in dilutions as low as 10^{-1} . The bacterial inhibition produced by cockle clams and abalone in the present study has been of a very low order when determined by the tube dilution method. Antibacterial activity was observed only in 10^{-7} and 10^{-8} dilutions of the test organism.

Enhanced growth in the form of larger colonies and denser growth was noted in many cases. This effect was noted with all three test organisms and may be due to enrichment of the medium by the extracts.

Burkholder, Burkholder and Almodovar (1960) have demonstrated the simultaneous production of growth-promoting and growth-inhibiting factors by Gonyaulax tamarensis. An extract of Gonyaulax, when applied to a nutrient-rich agar medium, inhibited the growth of S. aureus. When applied to a medium consisting only of sea water and agar and Gonyaulax extract enhanced the growth of various marine microorganisms.

The protection provided the E. coli C₂ bacteriophage may have been due to the presence of bivalent ions in the extract that were not present in the peptone water control. The addition of 0.005M magnesium sulfate to the peptone water protected the phage in the same manner as the extracts.

All extracts currently on hand will be tested by the tube dilution method. Further tests will be conducted employing summer samples of shellfish. Attempts will be made to purify extracts demonstrating activity by the tube dilution method. Tests against other bacteria and viruses will be conducted as cell lines are made available.

COMMENTS BY PARTICIPANTS

Dr. Matches inquired about the specificity of the agents found and whether or not the agent was heat stable, nondialyzable and of low molecular weight. Mr. Jakubowski replied that there was no specificity found and the agent was heat stable, nondialyzable and of low molecular weight according to Li.

Dr. Pringle discussed the relative information of whether or not the various fractions could be both antiviral and antibacterial. He suggested that gel diffusion techniques be used due to the nondiffusability noted by the paper disc method. Dr. Pringle also related some of the results that had been found by the Northeast Shellfish Sanitation Research Center in studies on the quahog clam. Very potent substances had been obtained from the crystalline styles but on dialysis part of this potency was lost. The question of artifacts on the use of chromatography was also mentioned. As the Northwest Shellfish Sanitation Laboratory did not have available equipment for chromatography or lyophilization no results could be compared at this time.

Dr. Quayle asked Dr. Pringle if the styles in quahogs contained the spirochete Cristaspira, as some crystalline styles are loaded with spirochetes. Dr. Pringle replied that the styles must be from animals collected directly out of the bed. Also different fractions would have varying potency with interphases of no activity. When the entire animal is homogenized a wide variety of material was extracted which would not give good results.

Dr. Quayle suggested that since the razor clam has a large and stable style it may lend itself quite well to the study.

Mr. Jakubowski explained that styles of several species were now on hand and would be tested. He reported on a study where fresh whole crystalline styles demonstrated inhibition to algae, but, when dissolved no further inhibition was noted.

Mr. Dupuy asked if any investigations on hemolymph had been attempted. The answer was negative.

Mr. McMillin suggested that different species of marine biota be investigated. It was agreed that as the project developed other species would be included.

Dr. Liu discussed the pitfalls that one might encounter in differentiating toxicity from therapeutic activity. Much research on activity against viruses, tumors and bacteria has been done by colleges and industry.

REFERENCES

- Burkholder, P. R., L. M. Burkholder and L. R. Almodovar. 1960. Antibiotic activity of some marine algae of Puerto Rico. Bot. Marina. 2(1/2):149-156.
- Li, C. P. 1960a. Antimicrobial effect of abalone juice. Proc. Soc. Exp. Biol. and Med. 103:522-524.
- Li, C. P. 1960b. Antimicrobial activity of certain marine fauna. Proc. Soc. Exp. Biol. and Med. 104:366-368.
- Li, C. P., B. Prescott and W. G. Jahnes. 1962. Antiviral activity of a fraction of abalone juice. Proc. Soc. Exp. Biol. and Med. 109:534-538.
- Li, C. P., B. Prescott, W. G. Jahnes and E. C. Martino. 1962. Antimicrobial agents from mollusks. Trans. N. W. Acad. Sci. 24:504-509.
- Prescott, B. and C. P. Li. 1960. Abalone juice. Fractionation and antibacterial spectrum. Proc. Soc. Exp. Biol. and Med. 105:498-500.
- Schmeer, M. R. 1964. Growth-inhibiting agents from Mercenaria extracts: Chemical and biological properties. Science. 4(24): 413-414.

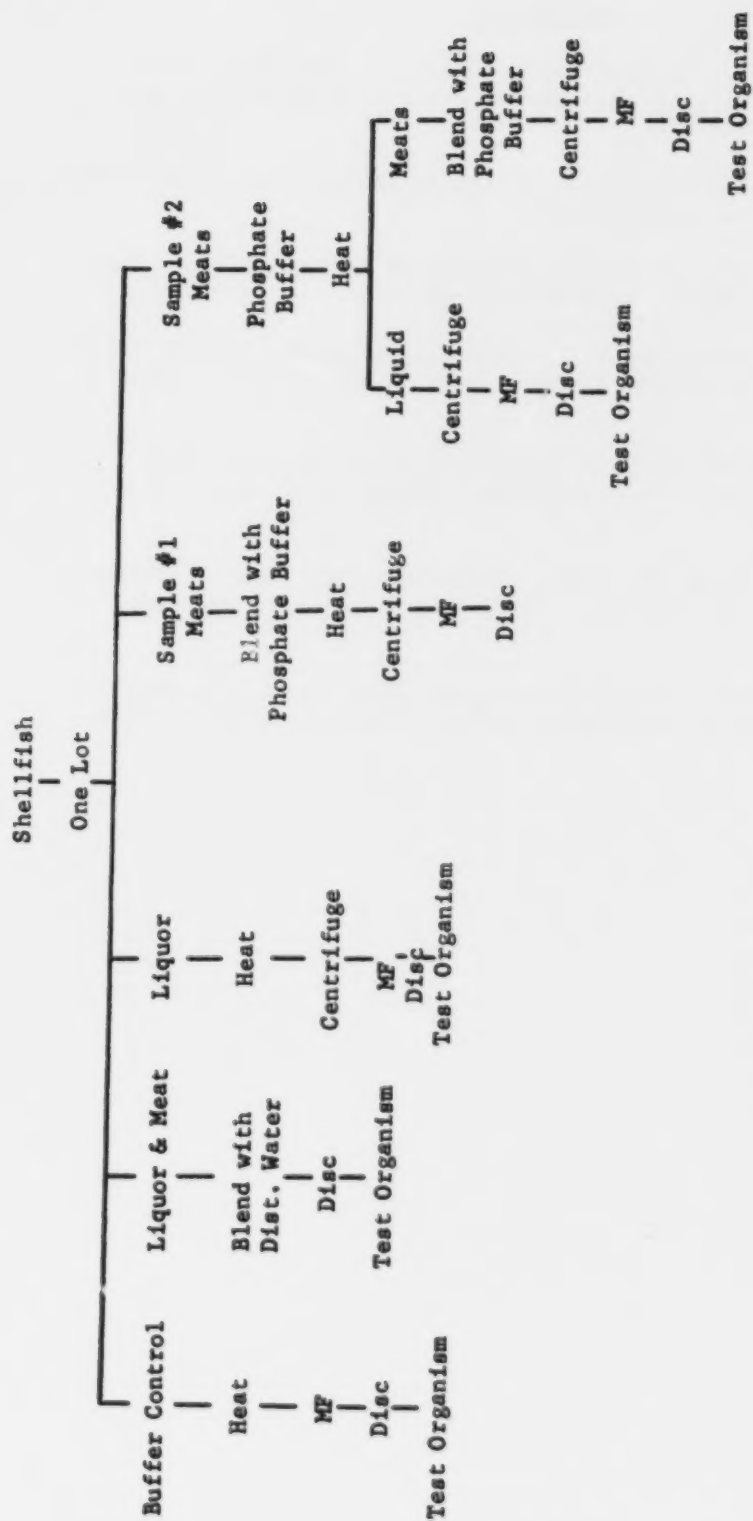


Fig. 1 Procedures used in preparation of extracts

Table 1. Species location by state

	<u>Washington</u>		<u>Oregon</u>		<u>California</u>	
	Available	Tested	Available	Tested	Available	Tested
Pacific oyster (<i>Crassostrea gigas</i>)	x	x	x	x	x	
Olympia oyster (<i>Ostrea lurida</i>)	x	x	x	x	x	
Kumamoto oyster (<i>Crassostrea gigas</i> , Kumamoto)			x	x		
Eastern oyster (<i>Crassostrea virginica</i>)					x	
Native Littleneck clam (<i>Protothaca staminea</i>)	x	x	x	x	x	
Butter clam (<i>Saxidomus giganteus</i>)	x	x	x		x	
Horse clam (<i>Schizothaureus nuttalli</i>)	x	x	x		x	
Manila clam (<i>Tapes japonica</i>)	x	x			x	
Softshell clam (<i>Mya arenaria</i>)			x		x	
Razor clam (<i>Siliqua patula</i>)	x	x	x		x	
Cockle clam (<i>Clinocardium nuttalli</i>)			x	x	x	
Pismo clam (<i>Tivela stultorum</i>)					x	
Bay mussel (<i>Mytilus edulis</i>)					x	

Table 2. Sampling localities by state

<u>Washington</u>	<u>Oregon</u>	<u>California</u>
North Sound	Tillamook	Crescent City
South Sound	Coos Bay	Humboldt Bay
Kitsap	Netarts Bay	San Francisco Bay
Hood Canal	Yaquina Bay	Monterey Bay
Straits	Seaside	Morro Bay
Grays Harbor		
Willapa		

Table 3. Colonial growth from 0.1 ml of broth culture containing extract plus 0.1 ml of serial dilution

Extract	Serial dilution of <i>S. aureus</i> (FDA 209)						0a
	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	
Phosphate buffer	con ^b	con	con	tnc ^c	0	0	0
Abalone juice	con	con	con	3,000	0	0	0
Abalone extract	con	con	0	0	0	0	0
Abalone viscera	con	con	con	con	0	0	3
Manila extract	con	con	con	con	0	0	0
Manila liquor	con	con	con	0	0	0	0
Razor liquor	con	con	con	0	0	0	0
Cockle extract	con	con	tnc	0	0	0	0

^aControl containing 1.8 ml BHI plus 0.2 ml extract

^bConfluent growth

^cToo numerous to count

Table 4. Effect of oyster and clam extracts on E. coli C2 bacteriophage^a

Shellfish	PFU/ml					
	Buffer Control	Untreated Liquor & Meats	Liquor	Meats	Blended Meats	Peptone water 0 min. 5 min.
Pacific oyster	163	-	123	124	111	
Native Littleneck	118	96	117	106	104	
Manila clam	107	-	111	129	107	102 31
Olympia oyster	103	-	93	86	98	

^a0.5 ml of each extract incubated 5 minutes with 0.5 ml of phage preparation containing 240 PFU/ml

RESEARCH ACTIVITIES AT OTHER PUBLIC HEALTH SERVICE
SHELLFISH SANITATION CENTERS

C. B. Kelly¹

The Shellfish Sanitation Research Centers at Narragansett and Dauphin Island have now been more than two years in full-scale operation. The year 1965 is characterized by the appearance of scientific and technical reports on the research and technical assistance activities of the Centers. To date, almost 40 reports have been prepared by the three Research Centers for presentation at scientific meetings. Many of these have been accepted for journal publication. The multi-disciplinary effort in our research program is evident from the scientific areas dealt with in these reports. The subjects include virology, parasitology, toxicology, chemistry and biochemistry. Technical assistance activities have been fruitful in the preparation of reports of surveys, field studies, and assistance to States in problems of unusual nature or magnitude.

The year 1965 was also marked by the Research Coordinating Conference at the Northeast Center, held May 5-6, 1965. This Conference was attended by 63 health officials, industry members and scientists from State agencies and universities. The Second Research Planning Conference was held by the Gulf Coast Research Center earlier this year and received similar attendance.

Highlights of the research and technical assistance activities of the Centers are:

Virus Research

Significant advances have been made in understanding the behavior of viruses in the estuary and the accumulation, retention and elimination of virus by shellfish. At Dauphin Island, Dr. John Mitchell and a team of scientists have completed studies on the Eastern oyster, Crassostrea virginica. The oysters were contaminated by exposure to seawater containing a constant level of the virus. In the accumulation phase of the experiments there was demonstrated a 50-fold accumulation of the virus by the oysters. When the polluted oysters were furnished ultraviolet-treated seawater, they freed themselves of the virus after 12 to 24 hours exposure. Dr. Liu and associates at the Northeast Center have investigated viral pollution and self-cleansing of the

¹Chief, Research and Investigations Section, Shellfish Sanitation Branch, Division of Environmental Engineering and Food Protection, Bureau of State Services, Public Health Service, Department of Health, Education and Welfare, Washington, D. C. 20201.

Northern hard clam, Mercenaria mercenaria.²

As in the Gulf Coast studies, the ability to accumulate virus was demonstrated and the viral polluted clams depurated within 48 to 96 hours. Dr. Liu's observations on organic localization of the virus is of particular significance. He demonstrated that most of the virus was confined to the digestive diverticula and that there was no penetration of organic cells. From the data presented there appears little doubt that theoretically, the digestive system can readily be cleansed within a reasonably short period of time.

Of particular significance also is the parallel behavior of bacteria and virus in the depuration process. This finding not only is reassuring for the success of depuration but also indicates that bacterial parameters may be used as indicators in control of the depuration process.

Meanwhile, Dr. John Hoff and his associates have worked on bacteriophage.³ In brief, the studies demonstrated persistence of the phage in seawater and in shell oysters for up to 20 days. He also demonstrated that the oysters rid themselves of phage in two to three days of depuration. In the course of these studies, he has developed and evaluated an extinction-dilution-quantitative method for phage in oysters.

Studies on Clostridium botulinum

Work on the occurrence of Clostridium botulinum in marine areas is in progress at all three Research Centers. You have heard reports on studies at the Northwest Laboratory.⁴ At the Northeast Center, Dr. Cabelli and his group are conducting comprehensive studies in two small estuaries in Rhode Island. Over three seasons of the year the organism was demonstrated in 7 to 19 percent of shellfish, marine and fresh-water sediments as well as terrestrial soil samples from the watersheds. Non-toxicological clostridia were isolated repeatedly from many of the ecological specimens. The studies are continuing as well as the development of improved methodology.

Parasitology

Dr. Cheng, at the Northeast Center, has undertaken a number of investigations in parasitology in shellfish and marine estuaries. Of interest are his studies on the pathological effect of infestation of two shellfish parasites, the trematode Bucephalus and the cestode Tylocephalum. These have been shown to produce extensive damage to

²A summary of the results of these experiments appears on Page 73 of this report.

³Reports on Dr. Hoff's work will be found on pages 74-91 of this report.

⁴See pages 58-70 of this report.

shellfish and to interfere with normal physiological functions of the shellfish. In other investigations he studied the potential for infestation of clams and oysters by Angiostrongylus cantonensis, a nematode whose infectious form is pathological to man. He was able to introduce infestation in the mollusc only under extremely unusual environmental situations.

Chemistry

The major effort in chemistry during the past year was in pesticide and heavy metal research. At the Northeast Center, Dr. Pringle and his associates have developed methods for toxic heavy metals in seawater and shellfish, using the atomic absorption spectrophotometer. These methods were then applied in studies of the presence of heavy metals in shellfish in Raritan Bay and in selected estuaries on the Atlantic Coast.

Studies of pesticides in shellfish have been conducted by the Gulf Coast Center after developing highly specific and sensitive methods for analysis of muds and shellfish by simultaneous microcoulometric and electron capture gas chromatography. The Center, in cooperation with the PHS Water Supply and Pollution Control Agency and the Departments of Health and Conservation, conducted intensive studies in the Mississippi Delta shellfish growing areas in Louisiana. These studies have shown low levels of chlorinated hydrocarbon pesticides in oysters, lower than those found in a recent "market basket" survey. The levels found in the "market basket" survey were declared to be of no public health significance.

Depuration

In addition to the laboratory research, the Centers have collaborated with State agencies in pilot and semi-commercial scale investigations using guidelines developed in studies conducted by the Maine Department of Sea and Shore Fisheries. The use of ultraviolet irradiation to sterilize seawater was initiated at Newburyport, Massachusetts, using the Purdy-type unit in one of their commercial tanks. Water was recirculated. Soft clams were found to cleanse themselves within 48 hours. The Bureau of Marine Fisheries, New York State Conservation Department has initiated pilot studies on clams at a shellfish plant at West Sayville, L. I.

The Shellfish Branch is currently preparing for an International Conference on Depuration to be held in July, 1966 at Narragansett, R. I. This conference will be of four days duration and will consider the technical and administrative problems in depuration of shellfish.

Technical Assistance Activities

A complete list of technical assistance activities is much too long to present in this report. It has represented a major effort in the activities of all of the Research Centers. It has been possible for us to respond somewhat to the needs of the Regional Offices, several State agencies and National Shellfish Sanitation Program.

Examples of these activities include:

- a) Development of oceanographic methods adaptable to estuarine sanitary surveys. Developed primarily on the Medomac River in Maine.
- b) Development and application of methods for shellfish resource surveys of marine estuaries. (Raritan Bay and certain estuaries in Maine.)
- c) Technical assistance to:
 1. Maine, New York, Massachusetts, and New Jersey in pilot and experimental depuration facilities.
 2. Alabama in sanitary surveys of Mobile Bay.
 3. Texas in development of legislation on depuration and problems associated with mosquito control in Galveston Bay area.

Facilities

Planning funds were appropriated for expansion of the Northeast Shellfish Sanitation Research Center. Space will be provided for high safety laboratories for research with highly infectious or toxicant materials. Funds for construction have been requested in the FY 1967 Budget. If the program proceeds on schedule the facility will be completed in FY 1969.

Planning and site acquisition funds have been appropriated for a shellfish sanitation research center in the Pacific Northwest. In fact, one of the missions of this trip is to participate in evaluation of potential sites in the States of Oregon and Washington.⁵

COMMENTS BY PARTICIPANTS

Dr. Sparks was very encouraged by the fact that the new Research Centers were studying oyster histology, parasitology and pathology rather than just oceanography and bacteriology. Messrs. Kelly and Jensen replied that the fields mentioned by Dr. Sparks were not abandoned but that administrative problems created delays and made it difficult to set up the programs with the right personnel. Mr. Kelly noted that the understanding of the oyster or clam was necessary to understand depuration.

⁵Later, the site at Manchester, Washington was selected by the Secretary of the Department of Health, Education and Welfare

PRELIMINARY ECOLOGICAL STUDIES ON THE PRESENCE OF
C. BOTULINUM

T. H. ERICKSEN AND W. J. BECK

INTRODUCTION

Previous studies have indicated Clostridium botulinum Type E to be associated with the marine environment (Dolman, 1957a; Dolman 1957b; Dolman and Iida, 1963). Because of the possible presence of C. botulinum Type E in the marine environment and the filter-feeding process of shellfish, the association of this organism with shellfish growing waters is of significance as a possible public health hazard. Therefore, this study was initiated to determine the presence of C. botulinum Type E in estuarine mud, terrestrial soil and shellfish samples from various sites in the Pacific Northwest. Samples for this study were obtained within the State of Washington. After familiarization with detection and isolation techniques using known C. botulinum Type E cultures, samples of mud, soil and shellfish were obtained from natural sources.

MATERIALS AND METHODS

Sampling:

Samples were collected with the cooperation of the Washington State Department of Health. Estuarine mud, terrestrial soil and shellfish were collected from the same immediate area where possible. Estuarine mud samples were obtained from sites which were covered by salt water at some time during the tidal cycle. Terrestrial soil samples refer to those obtained above higher high water. Estuarine mud and terrestrial soil samples were collected aseptically, placed in sterile screw-cap jars, and transported in crushed ice to the laboratory. Mud and soil samples were obtained to a depth of six inches. Where clam samples were obtained, associated mud samples were collected to the depth at which the clams were located. Shellfish samples were placed in plastic sacks and transported to the laboratory in crushed ice. All shellfish samples were examined immediately upon arrival at the laboratory. Mud and soil samples were either examined immediately upon arrival at the laboratory or placed under refrigeration for later examination. All mud and soil samples were examined within three days after collection.

Sampling Areas:

Areas where samples were collected are shown in Fig. 1. Sampling sites were chosen to give a cross section of the Puget Sound area as well as other major areas in Washington where shellfish are found.

A description of the sampling areas and types of samples collected is given below:

1. Meadow Point: - A polluted area with no commercial shellfish activity located on the northern shore of Shilshole Bay north of Seattle, Washington. Estuarine mud and a Pacific oyster sample were obtained from this site.
2. Middle Point: - An unpolluted area with no commercial shellfish activity. Located north of the village of Manchester, Washington. Estuarine mud, terrestrial soil, and one Pacific oyster sample were collected from the north shore of the point. One Manila clam and an accompanying mud sample were collected from the south shore of the point.
3. Budd Inlet: - A polluted area with no commercial shellfish activity. The sampling site was located on the west shore north of Olympia, Washington. Native Littleneck clam, butter clam and accompanying mud samples were collected.
4. Grays Harbor: - A large estuary located west of Aberdeen, Washington. There is limited commercial shellfish activity in this area. The sampling site was located near a small commercial oyster company south of Westport, Washington. Estuarine mud, terrestrial soil and Pacific oyster samples were collected.
5. Willapa Bay: - A large unpolluted estuary with considerable commercial shellfish activity located on the Pacific Coast in southwestern Washington. Mud, soil, Pacific oysters and Manila clams were collected from a commercial oyster bed near Nahcotta, Washington. A razor clam sample and an accompanying sand sample were also collected at Ocean Park, Washington.
6. Retail Markets: - Additional Native Littleneck and Manila clam samples from unknown sources were obtained from various retail markets in Seattle, Washington.

7. Burley Lagoon: - A commercial shellfish growing area located at Purdy, Washington. The sampling sites in the lagoon and its tributary fresh water streams are shown in Fig. 2. Estuarine mud samples were collected at the mouth of Purdy Creek; approximately 200 yards north of the mouth of Purdy Creek; around the island off the eastern shore of the lagoon; and in the upper lagoon mud flat. Fresh water streambed samples were collected from stations 1A, 11, 13, 20 and 21. Manila clams and associated mud samples were obtained at the mouth of Purdy Creek. Pacific oysters were collected north of the mouth of Purdy Creek along with associated mud samples.

Bacteriological Methods:

The procedures used for the determination of the presence of C. botulinum toxin and the subsequent identification of the type of toxin present were those of Eklund and Poysky (1965). An outline for the screening test for the detection of botulism toxin is given in Fig. 3. Mud, soil or shellfish homogenate were added to one-half pint milk bottles containing 75 ml of cooked meat enrichment medium. The cooked meat medium was formulated as outlined by Dolman (1957a). However, instead of using fresh meat, a commercial cooked meat medium was used as the basic medium. The bottles were incubated anaerobically in either Brewer or Case anaerobic jars at 25 C. Anaerobic incubation conditions were obtained by replacement of the atmosphere by either hydrogen from hydrogen generator envelopes (Brewer jars) or a mixture of 95 percent nitrogen and 5 percent carbon dioxide (Case jars). To determine the effectiveness of the replacement process a test tube containing a mixture of sodium bicarbonate, glucose, methylene blue and water was placed in each jar. After incubation, supernatant fluids were tested for mouse toxicity as shown in Fig. 3. Mouse toxicity tests were carried out using 20 g. Swiss Webster white mice. All tests were performed in duplicate. The mice were observed closely for the first 12 hours following injection for deaths due to nonspecific causes. All mice were held for four days.

Samples positive in the screening test were tested for the type of toxin present by neutralization with type specific antisera. An outline of the procedures used for this test is shown in Fig. 4. Types A, B and E C. botulinum antitoxins for neutralization tests were obtained from the Communicable Disease Center, Atlanta, Georgia.

RESULTS

This study indicated that the use of either Brewer or Case anaerobic jars supplied satisfactory anaerobic incubation conditions. However, the Case jar allowed considerably more samples to be incubated at one time. Close observation of inoculated mice immediately after inoculation indicated very few nonspecific deaths.

A summary of the results of a total of 41 estuarine mud and terrestrial soil samples is given in Table 1. Positive estuarine mud samples were obtained from Meadow Point (1), Middle Point (1), Burley Lagoon off the western tip of the island (1), Grays Harbor (2), and Willapa Bay (2). A positive streambed sample was obtained from Station 13 located on Burley Creek. Positive terrestrial soil samples were obtained from Middle Point (1), and Willapa Bay (1).

A summary of 20 shellfish samples tested to date is given in Table 2. Although some shellfish samples were collected from sites at which accompanying mud samples were positive, none of the shellfish samples were positive.

A preliminary experiment on the possible accumulation of C. botulinum Type E spores by Manila clam (Tapes japonica) shellstock has been carried out. In this experiment, the effect of dry storage at 3 C, 10 C and 20 C on the possible outgrowth of spores and toxin production in the exposed clams was studied.

The results of this experiment indicated that although the C. botulinum Type E spores were accumulated by the clams and the spores remained viable during storage at all three temperatures, there was no toxin production under these storage conditions.

DISCUSSION

The results of this limited survey have indicated C. botulinum Type E to be present in some estuarine mud and terrestrial soil samples collected from widely separated marine locations in the State of Washington. Other studies have also reported isolations of C. botulinum Type E from soil and mud in British Columbia (Dolman and Kerr, 1947), Japan (Nakamura et al., 1956), Denmark and Greenland (Pedersen, 1955) and the United States Pacific Northwest (Eklund, 1964). Clostridium botulinum Type E has also been isolated from fish (Prevot and Huet, 1951; Dolman and Chang, 1953; Nakamura et al., 1956) and from marine mammals implicated in botulism outbreaks (Dolman and Iida, 1963). Fish have also been implicated in C. botulinum Type E outbreaks in Denmark, Sweden and Russia (Dolan and Iida, 1963). Thus a wide geographical distribution of this organism in association with the marine environment is indicated.

In view of the above and the incidence of positive results shown by the soil and mud samples in this investigation, the negative findings in the associated shellfish were somewhat surprising. An isolation of C. botulinum Type E from the Eastern soft-shell clam (Mya arenaria), has been reported (Cabelli et al., 1965). Other reports of C. botulinum isolations from molluscs have not been found. Results of the preliminary accumulation and storage experiment indicate that if C. botulinum Type E were present in the concentration used experimentally, detection of the organism in clam meats would be possible.

This study indicated C. botulinum Type E to be present in some soil and mud samples from estuarine areas. However, it was not found on repeated samplings from the same locations in one area. Therefore, a more extensive study of this area will be made to determine the possible intermittent incidence of C. botulinum Type E. It may be possible to relate the influence of environmental factors to its presence. Further studies will also be made on conditions necessary for outgrowth and toxin production by C. botulinum Type E in various artificially polluted Pacific Coast shellfish.

REFERENCES

- Cabelli, V. J., I. Anderson, N. L. Richards, W. P. Hefferman, and G. P. Hoskins. 1965. Progress Report: Clostridium botulinum in the estuarine environment. Northeast Shellfish Sanitation Research Center; Narragansett, R. I.
- Dolman, C. E., and D. E. Kerr. 1947. Botulism in Canada, with report of a Type E outbreak at Nanaimo, B. C. Can. J. Pub. Health, 38:48-57.
- Dolman, C. E., and H. Chang. 1953. The epidemiology and pathogenesis of type E fishborne botulism. Can. J. Pub. Health. 44:231-244.
- Dolman, C. E. 1957a. Recent observations on type E botulism. Can. J. Pub. Health. 48:187-198.
- Dolman, C. E. 1957b. Type E (fish-borne) botulism: A review. Jap. J. Med. Sci. Biol. 10(b):383-395.
- Dolman, C. E. and H. Iida. 1963. Type E botulism: its epidemiology, prevention and specific treatment. Can. J. Pub. Health. 54:293-308.
- Duff, J. T., G. C. Wright, and A. Yarinsky. 1956. Activation of Clostridium botulinum type E toxin by trypsin. J. Bacteriol. 12:455-460.
- Eklund, M. W. (1964) Personal communication.
- Eklund, M. W. and F. Poysky. 1965. Clostridium botulinum Type F from marine sediments. Science, 149(3681):306.
- Nakamura, Y., H. Iida, K. Saeki, K. Kanzawa, and T. Karashimada. 1956. Type E botulism in Hokkaido, Japan. Jap. J. Med. Sci. Biol., 9:45-58.
- Pedersen, H. O. 1955. On type E botulism. J. Appl. Bacteriol. 18:619-628.
- Privot, A. R., and M. Huet. 1951. Existence en France du botulisme humain d'origine pisciaire et de Cl. botulinum E. Bull. Acad. Nat. Med., Nos. 25 and 26:432-434.

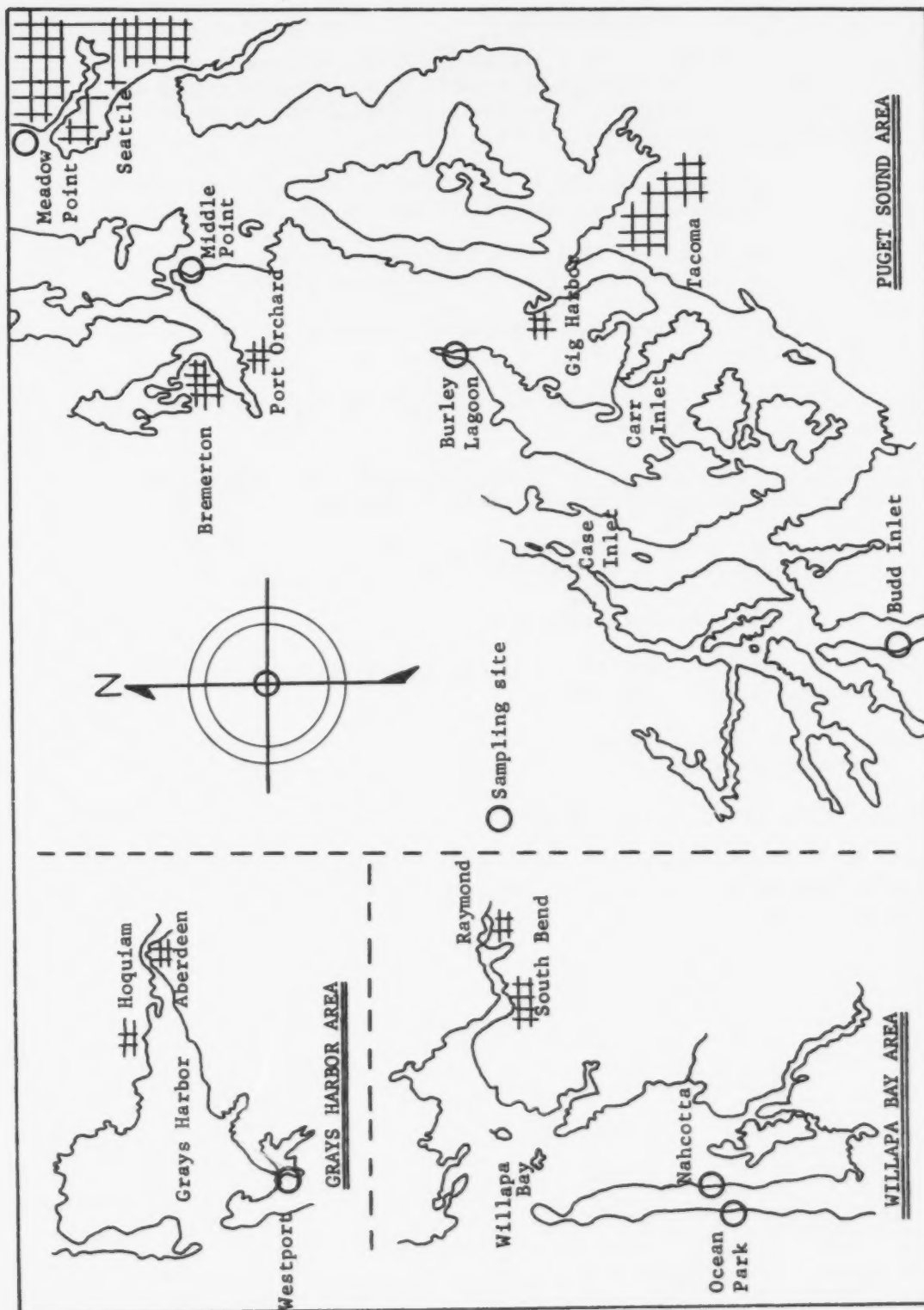


Fig. 1. Sampling sites in Puget Sound, Willapa Bay and Grays Harbor

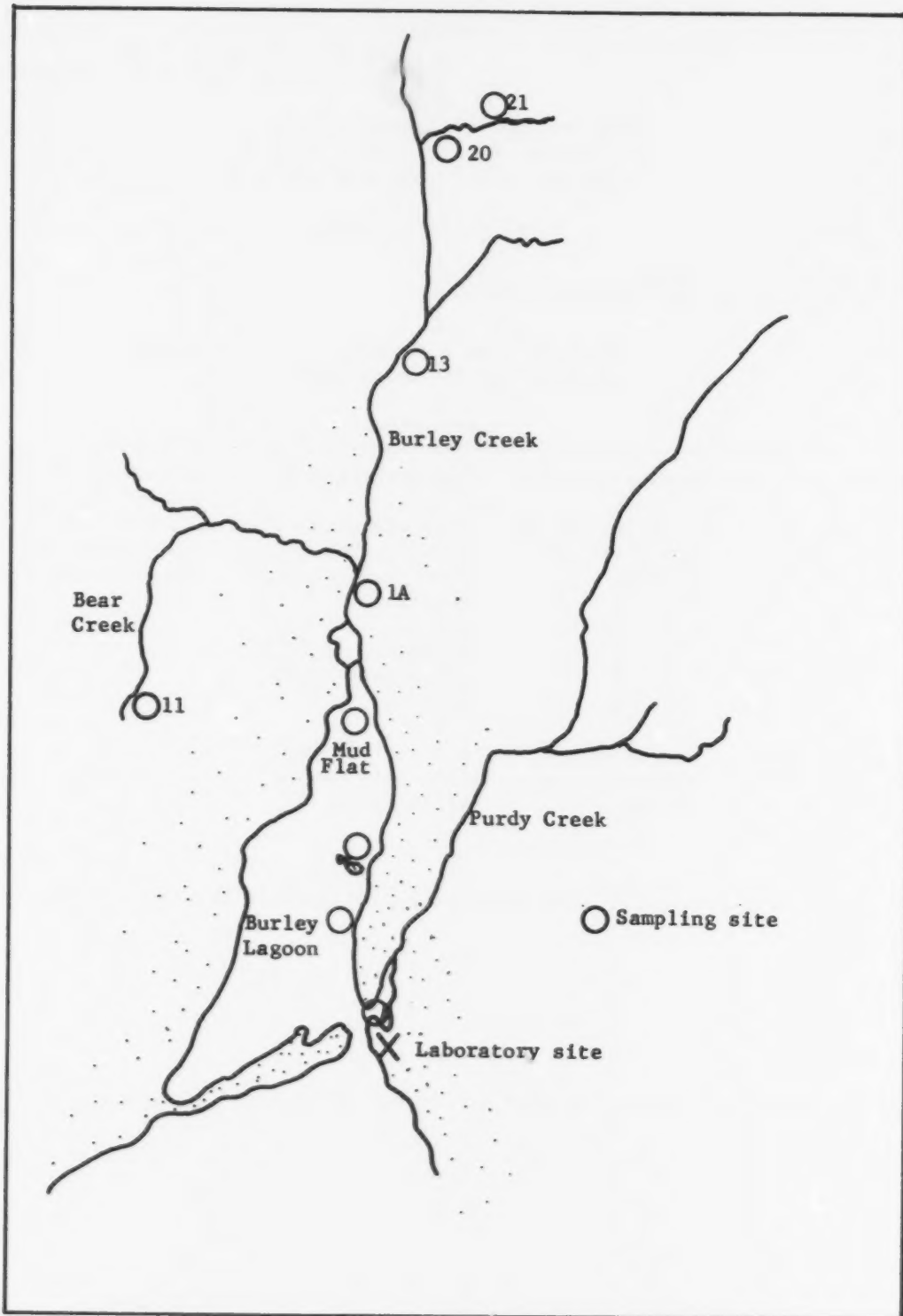
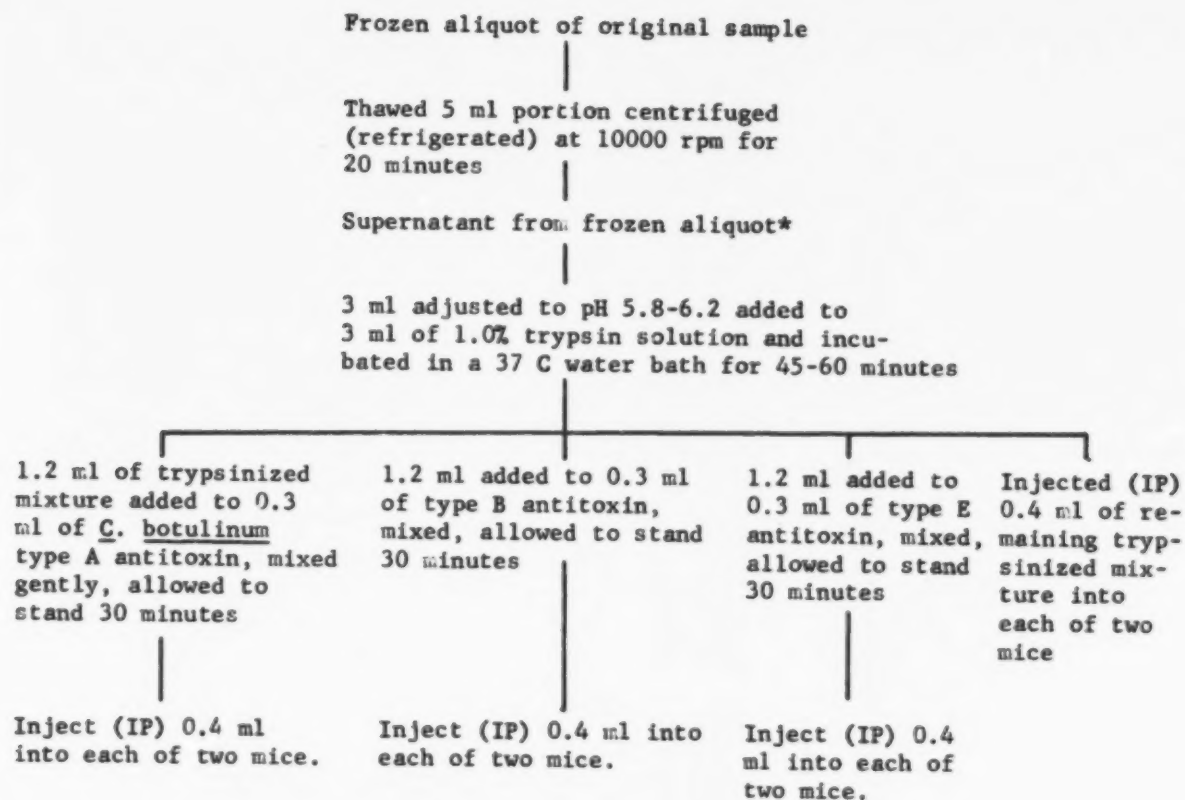


Fig. 2. Sampling sites in Burley Lagoon and its contributing watershed



All inoculated mice observed for 4 days and results recorded.

Fig. 3. Outline of Screening Test for Detection of Clostridium botulinum toxin



All inoculated mice observed for four days and results recorded.

*Refrigerated supernatant from the screening test may be used, however, only if a screening test was positive within 16 hours after the initial inoculations.

Fig. 4. Outline of Procedures for Detection of Types of Clostridium botulinum toxin

Table 1. Results of screening and subsequent neutralization tests on mud and soil samples collected to date

Area	Estuarine Mud Samples		Terrestrial Soil Samples		Ocean Mud Sample	
	No. of Samples	No. Positive for Type E	No. of Samples	No. positive for Type E	No. of Samples	No. positive for Type E
Meadow Point	1	1				
Middle Point	2	1	1	1		
Burley Lagoon and tributary streams	28	2				
Budd Inlet	1	0				
Grays Harbor	3	2	1	0		
Willapa Bay	2	2	1	1	1	0
Total	37	8	3	2	1	0

Table 2. Results of testing for botulism toxin in shellfish

Area	Pacific Oysters			Manila Clams			Native Clams			Butter Clams			Razor Clams		
	No. Samples	Type	No. Type E	No. Samples	Type	No. Type E	No. Samples	Type	No. Type E	No. Samples	Type	No. Type E	No. Samples	Type	No. Type E
Meadow Point	1		0												
Middle Point (north)	1		0												
(south)							1		0						
Burley Lagoon	1		0	3		0									
Budd Inlet (Mud Bay)							1		0	1		0			
Grays Harbor (south of Westport, Wash.)	1		0												
Willapa Bay (Nahcotta, Wash.) (Ocean Park)	1		0	1		0							1		0
Seattle Retail Markets	-		-	5		0	2		0	-		-	-		-
Totals	5		0	9		0	4		0	1		0	1		0

INCIDENCE OF CLOSTRIDIUM BOTULINUM ON THE PACIFIC COAST OF
THE UNITED STATES

Melvin W. Eklund
Bureau of Commercial Fisheries Technological Laboratory
Seattle, Washington

The incidence of Clostridium botulinum (with special reference to Type E) has been studied in Dungeness crab, crab processing plants, marine muds, trawl fish, and fish processing plants on the Pacific coast of the United States.

Incidence of Cl. botulinum in Dungeness crab

A total of 919 samples of Dungeness crab has been examined from the coastal areas of Alaska, Washington, Oregon, and California for the presence of Cl. botulinum.

The incidence of Cl. botulinum from Dungeness crab from the area of Ketchikan, Alaska was 57 percent; from Washington, 61-75 percent; from Oregon, 42-87 percent; and from California, 12-30 percent. The incidence of Cl. botulinum in Dungeness crab was lowest in the California area. However, it was also observed that the bacterial flora from these California samples was much more active in the production of proteolytic enzymes as evidenced by the action on the meat particles and also by the very high alkalinity encountered in the samples after incubation. Therefore, the low incidence of Cl. botulinum as demonstrated by the enrichment technique could in part be attributed to the possible destruction of the toxin by the proteolytic enzymes. This degree of proteolysis observed in the California samples was infrequently observed in the samples taken from Oregon, Washington, and Alaska.

The types of Cl. botulinum demonstrated from crab have been mainly Type E. Samples of crab from the Washington and Oregon coast have yielded Type B which are believed to be nonproteolytic in that trypsin activation was required to obtain lethal levels of toxin for mice. One sample of crab taken from the coast of Washington contained Cl. botulinum Type A. One sample of crab shell taken from the coast of California (San Francisco) contained the toxin of Cl. botulinum Type C.

Incidence of Cl. botulinum in Crab Processing Plants

A total of 164 crab plant samples (swabs of processing equipment, brine, and picked meat) have been examined for the presence of Cl. botulinum. With this limited number of samples, we have not been able to demonstrate the presence of Cl. botulinum in the plants beyond the boiling tanks. Research in this area is currently under study.

Incidence of *Cl. botulinum* in Marine Muds

A total of 273 mud samples have been examined for the presence of *Cl. botulinum*. Samples have been collected from Lake Washington (Seattle) and along the West Coast of the United States from Washington to the Mexican border.

The presence of *Cl. botulinum* Type E has been demonstrated in mud samples along the West Coast to 34° North Latitude; however, we were unable to demonstrate the presence of the organism south of 34° North Latitude. It has also been observed that more Type A *Cl. botulinum* were found in the California marine sediments than in the more northern waters.

Two cultures of *Cl. botulinum* Type A demonstrated in mud samples from The California coast required trypsin activation or obtain lethal doses of toxin. It is possible that these cultures are nonproteolytic because trypsin activation was required.

Two cultures of *Cl. botulinum* Type F have been demonstrated from the Oregon and California coast. These cultures are the first of this type to have been reported in the United States and the second time that this type has been isolated in the world. These cultures of Type F were demonstrated from mud samples taken at depths of 725 and 900 fathoms.

Cl. botulinum Type B has been demonstrated in marine muds from the Washington and Oregon coast. These are believed to be nonproteolytic in that lethal doses of toxin were demonstrated only after trypsin activation. Dr. Dolman reported that a nonproteolytic Type B was implicated in an outbreak of botulism from fermented salmon eggs, the first of its type to be reported from the North American continent.

Incidence of *Cl. botulinum* in Trawl Fish

A total of 271 samples of trawl fish has been examined for the presence of *Cl. botulinum*. The incidence of the botulinus organism in fish ranges from 0 to 100 percent. The higher incidence occurred in Rex sole taken from the mouth of the Columbia River and the source of the organism (one Type B and the remainder Type E) was mainly from the intestinal tract of the fish. The intestines were full of feed.

Fish taken from the other areas of Oregon and Washington however yielded lower incidence of 0 to 26 percent. The main sources of the cultures were from the intestines, gills, and swab of the fish surface.

Incidence of *Cl. botulinum* in Fish Processing Plants

Approximately 100 swab samples have been examined from the filleting part of a processing plant currently using in-plant chlorination. None of these samples have yielded cultures of *Cl. botulinum*. However,

one must recognize the fact that many other bacteria, many of which are proteolytic, are present and may be inhibitory to Type E or may destroy the formed toxin. Research in this area is currently under study.

Comparison of the Incidence of Type E in Mud, Crab, and/or Fish Taken in the Same Area

In several experiments, mud, crab, and/or fish were collected from the same area. There appears to be a close correlation between the incidence of Cl. botulinum Type E in crab and mud samples, but the incidence of Type E in fish and mud samples was highly variable.

COMMENTS BY PARTICIPANTS

Dr. Quayle asked about depth of mud samples. Dr. Eklund replied that the top 2 inches of the core were used.

Mr. McMillin was interested in whether or not any warm blooded marine animals had been tested. The answer was negative.

DEPURATION OF POLIOVIRUS POLLUTED CLAMS

O. C. Liu

Director, Marine Virus Research, Shellfish Sanitation Branch
Northeast Shellfish Sanitation Research Center
Narragansett, Rhode Island

The viral pollution of shellfish and their self-cleansing mechanism have been investigated by using the Northern Quahaugs and a strain of attenuated poliovirus I as the working model. The process of viral pollution of shellfish appeared to be a continuous one regardless of the level of virus pollution in surrounding sea water. The maximum uptake of virus in the drained fluids of the shellfish was achieved within four hours of pollution, whereas that in the digestive system achieved in 24 hours. The other organs of shellfish contaminated with very little virus under these conditions. The high plateau of viral contamination in these two former organs was maintained as long as there was sufficient virus present in the surrounding water. The virus harbored in the digestive diverticulum and stomach was found as not being adsorbed onto nor penetrating any types of cells. There is no evidence to indicate that any amount of viral multiplication has occurred in these organs.

When these viral contaminated shellfish were transferred into aquaria with running through sea water sterilized by ultraviolet treatment, the viral contaminants were cleansed to nondetectable level within 48-96 hours. It appears also from the present data that the lightly polluted shellfish required shorter time to be cleansed than those which were polluted heavily. It is reasonable to assume that the naturally polluted shellfish probably contain even less virus than the lightly contaminated ones as described in these experiments.

Theoretically, self purification process of the naturally contaminated shellfish may be completed very rapidly under appropriate conditions.

COMMENTS BY PARTICIPANTS

Mr. Foster inquired as to what viruses were used in this study. Dr. Liu replied that poliovirus I, II and III were used at this time.

Mr. McMillin wanted to know if the virus in the clam feces was as virulent as before. Dr. Liu stated that the viruses acted the same. He felt that the virus only passes through the animal and was not associated with the cells of the animal.

Messrs. Dupuy and Foster were interested in the ability to tag viruses with radioactive isotopes. Dr. Liu replied that some viruses might be killed but the use of an in Vivo test with proper calculation of ratios would determine this.

STUDIES ON BACTERIOPHAGE ACCUMULATION AND ELIMINATION BY THE PACIFIC
OYSTER (CRASSOSTREA GIGAS)

J. C. Hoff, W. Jakubowski and W. J. Beck

INTRODUCTION

A bacteriophage was used as a model virus to study the accumulation and elimination by shellfish of biological entities smaller than bacteria. The results presented previously (Hoff and Beck, 1964) showed that, under the experimental conditions employed, phage levels accumulated in the oyster meats were much lower than those in the shell liquor and overlying water. Depuration of the polluted oysters proceeded rapidly but in shell oysters stored dry at 5 C, the phage was quite stable.

However, these studies did not show conclusively whether the low accumulation levels were due to failure of the oysters to accumulate the particles or to inactivation subsequent to accumulation. These investigations were made in efforts to obtain more detailed information on the fate of these particles in Pacific oysters.

MATERIALS AND METHODS

Bacteriophage:

The phage was isolated from sewage using Escherichia coli C2 as the host bacterium. The phage is stable in seawater and is inactivated by ultraviolet radiation. The phage is 390 mu long overall and the head portion is 110 mu wide.

Oysters:

Pacific oysters (Crassostrea gigas) 10-15 cm high collected in upper Burley Lagoon, were used.

Media:

Media used for phage production were described by Groman and Suzuki (1962). Overlay agar consisted of 1.2% agar (Difco) in distilled water. Final agar concentration in the overlay was 0.6%.

Phage Assay Methods:

The agar overlay plaque method was described previously (Hoff & Beck, 1964). This method is unsatisfactory for assaying water samples containing phage concentrations of less than 20 PFU/ml. In the case of blended oyster meats, at least 200 PFU/ml must be present because the

samples must be diluted 1:10 to avoid masking of the plaques by meat particles.

A method employing the dilution endpoint or MPN principle was developed. The procedures used are described below. Sterile metal-capped tubes (18x150 mm) were placed in racks and 1.5 ml of a 3-4 hour broth culture of E. coli C2 was added to each tube. Tubes which were to be inoculated with 2.0 ml of a 1:2 dilution of blended oyster meats received 3 ml of culture. Tenfold dilutions of the sample were made and 1.0 ml of each dilution was placed in each of five tubes. After overnight incubation at 30 C the tubes were checked for phage multiplication. This was done by placing a loopful of culture from each tube on a Trypticase Soy Agar plate which had been previously dried¹ and spread with the phage-sensitive E. coli C2 culture. These plates were then incubated overnight at 30 C and examined for evidence of phage activity. Areas showing evidence of lysis of the confluent bacterial growth were considered positive (Fig. 1). A standard MPN table was used to convert the results into phage MPN/g or ml. The same samples were also examined by the plaque technique and the results were compared statistically.

Stationary Aquarium Accumulation-Elimination Experiments:

The small flat, 75 cm long, 20 cm wide, and 11 cm deep was used as the polluting aquarium and is shown in Fig. 2. Twenty oysters were placed in the aquarium and 10 liters of sea water were added. The water was circulated by an epoxy coated motor-driven propellor. One ml of phage preparation (3×10^{10} PFU/ml) was then added to give a final concentration of 3×10^6 PFU/ml in the seawater. Fresh seawater-phage preparations were added at 24-hour intervals. The oysters were held in the pollution phase 2-3 days. The depuration was carried out in larger flats in a flowing seawater system.

Oyster Feces and Pseudofeces Collection:

Plastic troughs (Fig. 3 and Fig. 4) separated longitudinally into two compartments were used to collect feces and pseudofeces. The oysters were placed directly over the dividing strip so that feces and pseudofeces would fall into the separate compartments. Prior to being placed on the collectors, the contaminated oysters were placed in unpolluted flowing seawater for 15-45 minutes to reduce the high phage concentration in the shell liquor.

¹Placed in 35 C incubator overnight to remove excess moisture from agar surface.

The oysters remained on the collectors for various periods of time. When feces and pseudofeces samples were to be collected the oysters were removed and the troughs were carefully lifted out of the aquarium. Samples of the overlying water remaining in both sides of the trough were taken. Phage assays on these samples indicated the possible extraneous or "background" phage contamination of the feces and pseudofeces samples. The excess water in the collectors was then decanted. Most of the water remaining after this procedure was removed by pipette, leaving approximately 2-3 ml of water and feces or pseudofeces deposited at the end of the trough. This material was thoroughly mixed by pipette and samples of feces and pseudofeces were examined separately for phage content.

Other Experiments:

It seemed likely that additional information on the fate of viruses in shellfish might be obtained by attaching the phage to a larger particle known to be accumulated by shellfish. Several attempts were made to do this using E. coli C2, the propagating strain for the phage. Because of failure to obtain good preparations of E. coli with phage attached, little was gained from these experiments. Since these experiments could be carried out only while water temperatures were low enough to prevent progression of the lytic cycle by the phages attached to E. coli they were discontinued in early spring.

Two other methods were used in attempts to introduce the phage into the interior of the oyster. One of these methods consisted of placing phage in the mantle cavity of live oysters. To accomplish this, wedges were placed between the valves of oysters. The oysters were then removed from the water and phage was placed in the mantle cavity using a 5 ml syringe and a 14 gauge cannula. The wedges were then removed and the oysters were stored dry at 5 C and 15 C. Samples of washed meats and liquor were assayed after various incubation periods.

Inoculation directly into the stomach was also attempted. To do this, portions of the left and right valves at the posterior end were cut away so that a hole approximately 1 cm in diameter was made adjacent to the stomach. Using a 25 gauge needle and a tuberculin syringe, 0.1 ml of phage suspension was injected into the stomach. The oysters were then placed on collectors in flowing U. V. treated seawater. Samples of meat, shell liquor, feces and pseudofeces were taken at intervals and assayed for phage content.

RESULTS

The results shown in Table 1 show that phage introduced into the mantle cavity was not accumulated to any extent by the oysters since nearly all of the phage remained associated with the shell liquor. The levels in the liquor appear to be lower than the concentration introduced. However, this was due in part to dilution since the meat and liquor results were expressed on a PFU/ml or g basis while the amount injected was the total number of PFU/oyster.

The results of an experiment in which phage was injected into the stomach are shown in Table 2. In this case the amount introduced was also on a total PFU/oyster basis while the assays were on a PFU/g or ml basis. As would be expected with this method, immediately after injection, phage concentrations associated with the meats were higher than in the shell liquor. The appearance of a high concentration in the feces one day after injection indicated that the phage was passing through the intestinal tract. However, the pattern of decrease shown by the meat samples was somewhat erratic, indicating that perhaps in some cases, the phage was being injected into tissue rather than into the intestinal tract.

Since these methods did not appear to be suitable for further studies on the fate of naturally ingested phage, further experiments were conducted in the stationary flats described above. The stability of the phage in seawater during these experiments is shown in Table 3. The results indicate that the phage was very stable in this system either in the presence or absence of oysters. These results also indicated that the oysters did not ingest sufficient phage to cause a detectable phage concentration reduction in the overlying water.

During the depuration phase of the stationary flat experiments, the endpoint dilution method described above was used to quantitate phage present in concentrations below the limits of the plaque method. Where possible both methods were used. The results of a comparison of phage MPN and plaque count results are shown in Table 4. A probability plot of individual MPN/plaque count ratios indicated that MPN results were somewhat lower than plaque count results. The 10, 50 and 90 percentiles were 0.26, 0.61 and 1.4 respectively. The range of MPN/plaque count ratios was from 0.15 to 3.04. Therefore, in the figures and tables below, results were based on plaque counts in the range where these were valid (based on 20 or more plaques/plate) and on MPN results when phage concentrations were too low to be enumerated by the plaque method.

Accumulation of phage in the stationary system is shown in Table 5. The results show that phage levels in both washed meats and liquor were as high after 15 minutes exposure as after 72 hours. However, shucked oysters which had been stored overnight in seawater containing 2×10^6 PFU/ml and then washed also showed levels similar to those in Table 5. This indicated that even after thorough washing phage concentrations on the exterior of the oyster body remained relatively high.

The results of depuration of oysters polluted for 48-72 hours in the stationary system are shown in Figs. 5, 6, 7 and 8. Fig. 5 shows the results of depuration for six days. Since depuration was not complete, two additional experiments were carried out over a 14-day period. The results of these studies are shown in Figs. 6 and 7. The depuration patterns shown in these three figures are very similar, all showing a rapid decrease in concentration in both washed meats and shell liquor. After the initial decline, levels in shell liquor remained about the same or slightly higher than those of the meats. As depuration time increased, the rate of decrease became less with phage still detectable in some individual oysters even after 14 days. A more detailed study of the first 48 hours of depuration is shown in Fig. 8. These results show that the initial depuration rate was even more rapid than shown in Figs. 5, 6 and 7.

Additional information on the elimination of phage is given in Tables 6 and 7. In these experiments the polluted oysters were placed on the collectors described above and feces and pseudofeces were assayed in addition to the oyster meats and shell liquor. The phage levels found in the feces indicate that phage had been ingested by the animals and had survived passage through the intestinal tract. Phage levels in the feces were highest during the early part of the depuration period but some phage was still found in feces collected between the 48th and 72nd hour. Phage was also present in pseudofeces but at lower concentrations than in the feces. The results of these analyses are shown graphically in Fig. 9.

DISCUSSION

The results of the phage accumulation studies indicate that maximum levels associated with the washed meats were attained very rapidly. It is possible that the greater part of the phage being detected in these washed meats may have been present on the external surfaces of the animal bathed in the polluted sea water or in areas such as the gill chambers and water tubes not affected by the washing process. However, results of another experiment, not shown above, did indicate that phage was ingested during a 15 minute exposure to pollution, since 24 hour feces samples from these animals contained considerable phage. In this connection, rapid accumulation of Escherichia coli by Crassostrea virginica was reported by Galtsoff and Arcisz (1954).

Previous studies on elimination of phage by oysters showed that phage levels in meats and shell liquor of depurating oysters dropped rapidly to levels below limits detectable by the overlay method. The use of high levels of phage pollution and the phage endpoint dilution method for phage quantitation made it possible to study elimination rates in more detail and to follow the reduction in phage concentration to much lower values.

Elimination rates followed the same general pattern in all of the experiments. In every case 99 percent of the phage had been removed from the meats after 24 hours depuration. In one case (Fig. 4) nearly 99 percent was removed within 2 hours. The reasons for the decline in elimination rates as depuration time progressed are not known. Phage levels in shell liquor declined rapidly to those of the meats. However, during the latter part of depuration, levels in shell liquor remained about the same or slightly higher than those of the meats. Again, the reasons for the presence of this residual phage is not known. It does appear that some of the phage was located in areas from which it was not readily removed during depuration.

The results of feces and pseudofeces examinations showed that most of the phage present in these excretions was removed during the first 24 hours. Lower levels of phage continued to be detected for the duration of sampling (72 hours). In contrast, Galstoff (1964) reported that food passed through the entire intestinal tract of large (10x6 cm) Eastern oysters in 90 to 150 minutes.

The examination of feces samples provided some additional information on the fate of phage in the Pacific oyster. The results show that phage ingestion did occur and that the phage survived the feeding process and passage through the digestive tract. Concentrations found in the feces were lower than the levels in the polluting water, suggesting that the phage was not being selected and concentrated by the oysters. However, several factors which may have influenced these findings should be pointed out.

It is possible that the figures are low because destruction of some of the phage may have occurred during passage through the intestinal tract. It is also possible that phage was released from the feces lying in the collecting troughs and washed away by the seawater moving through the collectors. The "background" phage counts indicate that loss by this method was slight. Also, the seawater was not completely removed from the trough containing the feces and pseudofeces. Some was allowed to remain and was used to suspend the feces. The effect of this would be to dilute the feces and therefore lower the phage concentration but it is difficult to estimate the degree of dilution. It would be difficult

to separate the oyster feces from the water since any treatment would likely break up the feces and release phage. Since the effect of all three of these factors would be to lower the results it can be assumed that phage levels in the feces were actually somewhat higher than those shown in these results.

Although phage was still detectable after 14 days depuration, it should be pointed out that the polluting concentrations used (3×10^6) were very high. Theoretical calculations by Clarke *et al* (1964) indicate that expected virus concentrations in domestic raw sewage would be about 700 virus units/100 ml and in polluted water 0.15 to 1.5 virus units/100 ml.

Also, in each case 99 percent of the phage was removed during the first 24 hours depuration. If elimination of naturally ingested virus occurs at rates similar to those shown for phage in this investigation, it appears that depuration would be a satisfactory method for removal of viruses from Pacific oysters.

COMMENTS BY PARTICIPANTS

Mr. J. Dupuy inquired into the use of aprons around individual oysters. Dr. Hoff reported that this had not been attempted. A discussion on methods of opening oysters in order to retain the hemolymph was made by Mr. Dupuy.

Dr. Matches asked about the consistency of feces and pseudofeces. Dr. Sparks stated that one should obtain more feces than pseudofeces in this area. More pseudofeces would be produced where turbidity in the water is high. Mr. Dupuy discussed the production of pseudofeces as related to algae. As there is a selective rejection the virus may be coming out in a pure string of mucous.

REFERENCES

- Clarke, N. A., G. Berg, P. W. Kabler, and S. L. Chang. 1964. Human enteric viruses in water: source, survival and removability, p. 523-542. In International Conference on Water Pollution Research London, Sept. 1962. Pergamon Press, Oxford, London, New York, Paris.
- Galtsoff, P. S. 1964. The American oyster (Crassostrea virginica) Gmelin. U. S. Dep. Int. Fish. Bull. 64. 480 p.
- Galtsoff, P. S. and W. Arcisz. 1954. Observations on the rate of propulsion of water and retention of coliform bacteria by the oyster. Nat. Shellfish. Assn., 1953 Convention Papers, pp. 1-8.
- Groman, N. B. and G. Suzuki. 1962. Temperature and lambda phage reproduction. J. Bacteriol. 84:431-437.
- Hoff, J. C. and W. J. Beck. 1964. Studies on the behavior of a bacteriophage in the Pacific oyster (Crassostrea gigas). Proc. N.W. Shell. Sanit. Res. Plann. Confer. PHS Pub. No. 999-FP-4.



Fig. 1 Spot plates for phage MPN determinations



Fig. 2 Stationary aquarium

Reproduced from
best available copy.



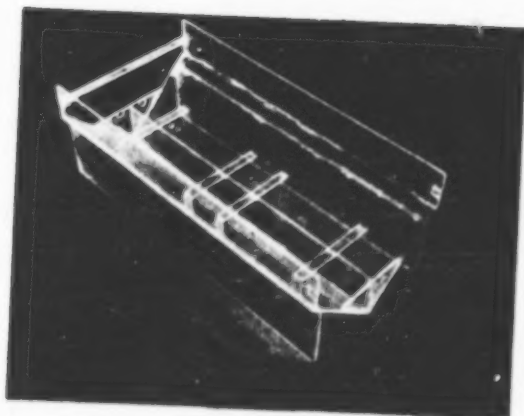


Fig. 3 Apparatus used for collection of feces and pseudofeces

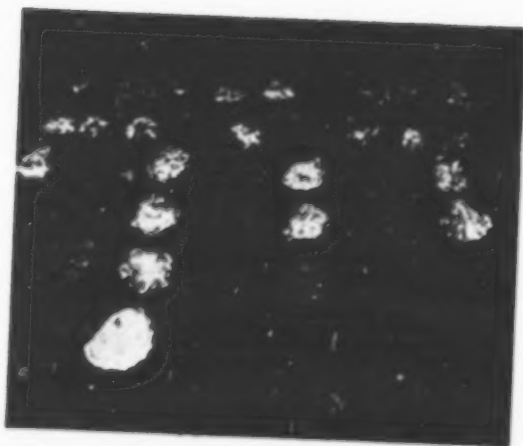


Fig. 4 Oysters in position on collectors

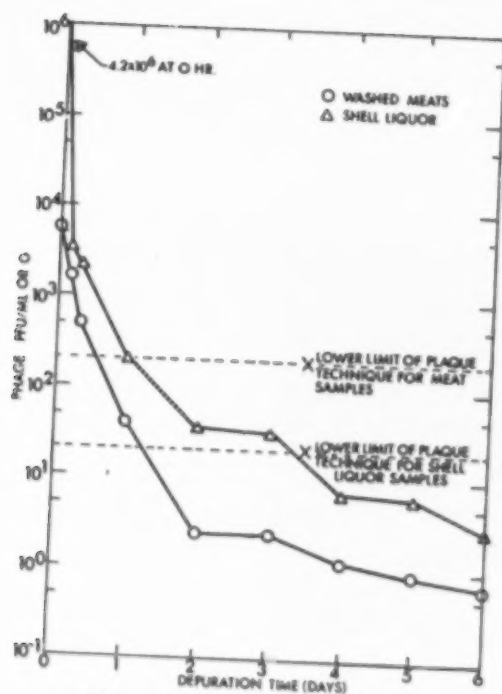


FIG 5 DEPURATION OF PACIFIC OYSTERS EXPOSED TO 4.2x10⁶ PFU/ML (EXP N)

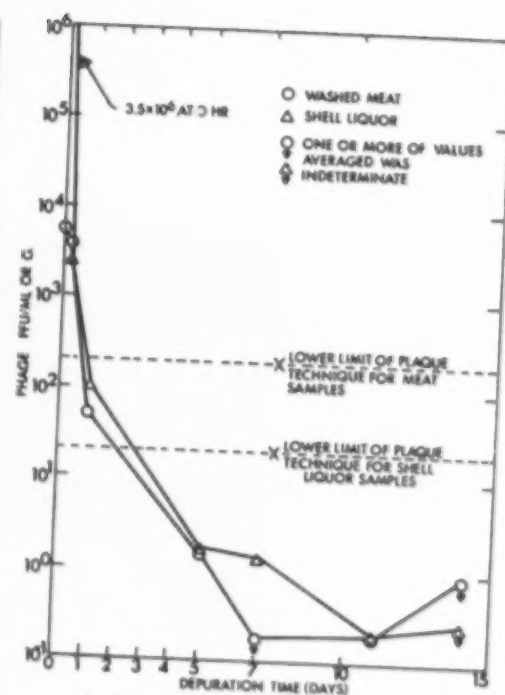


FIG 6 DEPURATION OF PACIFIC OYSTERS EXPOSED TO 3.5x10⁶ PFU/ML (EXP P)

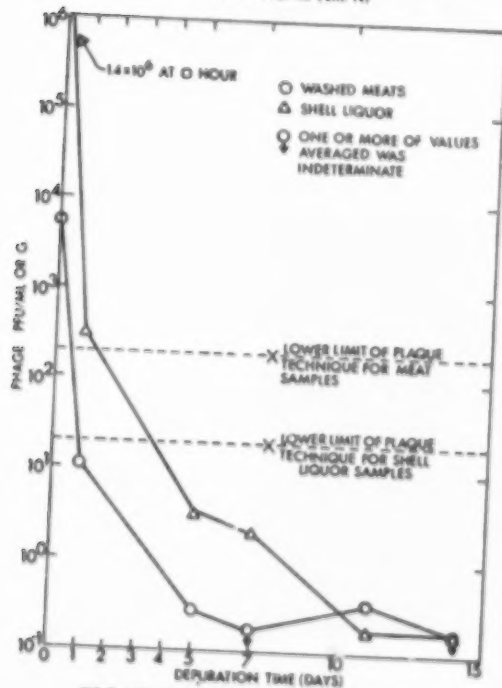


FIG 7 DEPURATION OF PACIFIC OYSTERS EXPOSED TO 1.4x10⁶ PFU/ML (EXP Q)

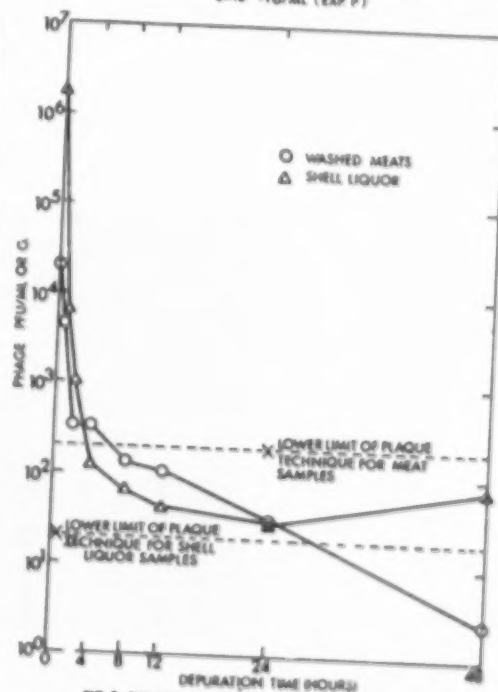


FIG 8 DEPURATION OF PACIFIC OYSTERS EXPOSED TO 3x10⁶ PFU/ML

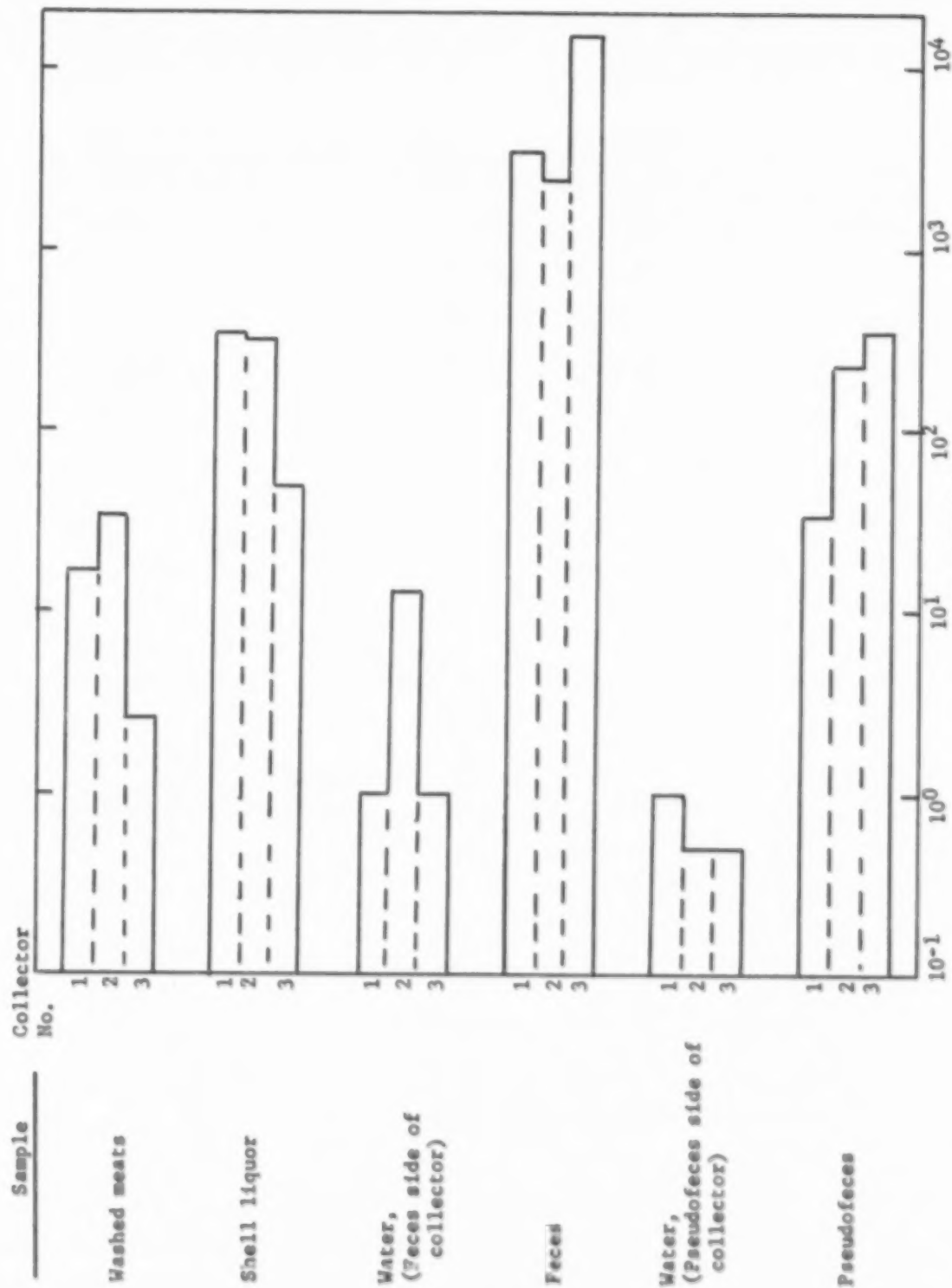


Fig. 9 Phage content of oysters and oyster excreta after 24 hours depuration

Table 1. Phage accumulation by contaminated^a oyster shellstock stored dry at 5 C and 15 C

Time after Contamination (days)	PFU/g or ml			
	5 C Storage		15 C Storage	
	Washed Meats	Liquor	Washed Meats	Liquor
0	8.5x10 ⁴	1.8x10 ⁸		
3	7.4x10 ⁵	1.1x10 ⁸	1.0x10 ⁵	2.5x10 ⁸
5	3.9x10 ⁵	1.4x10 ⁸	6.3x10 ⁵	1.3x10 ⁸

3.3x10⁹ PFU placed in mantle cavity of each oyster

Table 2. Depuration of oysters injected with phage (2.4x10⁸ PFU/oyster)

Depuration Time	PFU/g or ml			
	Meats	Liquor	Feces	Pseudofeces
0	6.9x10 ⁵	9.4x10 ³		
1 day			1.3x10 ⁶	8.3x10 ²
2 days			1.4x10 ⁴	1.3x10 ²
3 days	2.0x10 ¹ 1.5x10 ²	2.0x10 ⁰ 2.0x10 ⁰	8.0x10 ¹	2.0x10 ¹
4 days	9.9x10 ² 3.2x10 ⁴ 1.2x10 ³	2.0x10 ⁰ 8.0x10 ⁰ 3.0x10 ⁰	7.0x10 ¹	0

Table 3. Phage stability in seawater used to pollute oysters

Time ^a (hrs)	PFU/ml (x10 ⁶)	Time ^a (hrs)	PFU/ml (x10 ⁶)
0	1.5	0	3.6
26	2.4	24	2.9
0	4.3	0	2.7
23	1.6	24	3.2
0	4.3	0	3.8
25	4.3	4	3.4
0	3.0	0	5.3
17	3.4	19	3.1
96 ^b	2.9		

^aNumber of hours after addition of phage to water.
^bNo oysters present in aquarium after 17 hours.

Table 4. Comparison of phage plaque count and MPN results

No.	Type	Geometric Mean		Ratio: $\frac{\text{MPN}}{\text{Plaque Counts}}$
		MPN	Plaque	
13	Oyster meats feces and pseudofeces	2.4x10 ³	3.5x10 ³	0.69
17	Shell liquor and water	1.2x10 ⁵	2.1x10 ⁵	0.57
30	All samples	2.2x10 ⁴	3.5x10 ⁴	0.63

Table 5. Phage accumulation in washed meats and shell liquor following exposure to 3×10^6 PFU/ml for various time periods

Period of Exposure	PFU/g or ml	
	Washed Meats Plaque Count	Shell Liquor Plaque Count
15 minutes	2.4×10^3	4.1×10^6
	8.3×10^3	1.0×10^6
1 hour	1.6×10^4	2.4×10^6
4 hours	5.3×10^3	3.2×10^6
24 hours	7.0×10^3	3.0×10^6
	5.6×10^3	9.6×10^5
48 hours	5.1×10^3	2.2×10^6
	7.5×10^3	3.7×10^6
72 hours	4.3×10^3	4.8×10^6

Table 5. Elimination of phage in feces and pseudofeces of Pacific oysters

Depuration Time	Oysters ^a		Collected Feces and Pseudofeces				
	Washed Meats	Shell Liquor	Collection Period	Feces Side		Pseudofeces Side	
				Water ^b	Feces	Water ^b	Pseudofeces
0	5.3x10 ³	3.2x10 ⁶					
4 hr.	3.6x10 ³	2.5x10 ³					
24 hr.	4.9x10 ¹	7.9x10 ¹	4th-24th hr.	1.2x10 ¹	7.0x10 ⁴	2x10 ⁰	1.4x10 ³
48 hr.	-	-	24th-48th hr.	5x10 ¹	4.9x10 ¹	1x10 ¹	7.0x10 ¹
72 hr.	-	-	48th-72nd hr.	-	1.7x10 ¹	-	6.8x10 ⁰

^aExposed for two days to 3x10⁶ PFU/ml in stirred aquarium.

^b"Background" concentration (See methods and materials).

Table 7. Elimination of phage in feces and pseudofeces of Pacific oysters during early phase of depuration

Depuration Time	Oysters ^a		Examination of Feces ^b and Pseudofeces ^b				
	Washed Meats	Shell Liquor	Collection Period	Feces Side		Pseudofeces Side	
				Water ^c	Feces	Water ^c	Pseudofeces
0	2.0x10 ⁴	1.9x10 ⁶					
45 min.	4.5x10 ³	6.8x10 ³					
2 hr.	3.4x10 ²	1.2x10 ³	0 - 2nd hr.	3.0x10 ¹	1.8x10 ⁵	1.4x10 ¹	5.4x10 ³
4 hr.	3.4x10 ²	1.5x10 ²	2nd - 4th hr.	8.0x10 ⁰	1.1x10 ⁴	1.1x10 ¹	4.0x10 ²
8 hr.	1.5x10 ²	7.1x10 ¹	4th - 8th hr.	1.2x10 ¹	3.8x10 ³	1.0x10 ¹	3.7x10 ²
12 hr.	1.2x10 ²	4.7x10 ¹	8th - 12th hr.	5.0x10 ⁰	6.4x10 ²	3.0x10 ⁰	2.4x10 ²
24 hr.	3.4x10 ¹	3.0x10 ¹	12th - 24th hr.	3.0x10 ⁰	1.7x10 ²	2.0x10 ⁰	1.3x10 ¹
48 hr.	2.8x10 ⁰	8.0x10 ¹	24th - 48th hr.	-	3.3x10 ¹	-	7.9x10 ⁰

^aExposed for two days to 3x10⁶ PFU/ml in stirred aquarium.

^bPooled sample from six oysters.

^c"Background" concentration (See methods and materials).

STUDIES ON MEMBRANE FILTRATION OF BACTERIOPHAGE

J. C. Hoff and W. J. Jakubowski

INTRODUCTION

During studies on the accumulation and elimination of bacteriophage by Pacific oysters, sea water contaminated with phage was passed through membrane filters of 0.45u pore size. It was noted that although the phage concentration in the sea water prior to filtration was about 1×10^3 plaque-forming units (PFU)/ml, phage was not detectable in the filtrate. This was not expected since one step in the preparation of stock phage suspensions involved filtration through the same type of filter. Little loss of phage was noted in this procedure.

It appeared that this phenomenon might provide a basis for the development of methods for concentration and enumeration of phage or possibly viruses present in low concentrations in sea water or similar samples. Therefore, a limited investigation of several aspects of this phenomenon was made.

MATERIALS AND METHODS

Bacteriophage:

The phage was isolated from sewage using Escherichia coli C2 as the host bacterium. The phage is 390 mu long and 110 mu wide. It has been used in accumulation-elimination studies in the Pacific oyster (Hoff and Beck, 1964; Hoff, Jakubowski and Beck, 1965). Stock phage suspension prepared in 0.1% peptone water contained about 5×10^7 PFU/ml.

Media:

Media used for phage propagation were those described by Groman and Suzuki (1962). Overlay agar consisted of 1.2% agar (Difco) in distilled water. Phage diluent consisted of 0.1% peptone with 0.005MMgSO_4 in distilled water.

Several types of sea water were used as suspending media for the phage. These included: fresh sea water, filtered sea water (fresh sea water which had been filtered through HA 0.45u filters before use), aged sea water (stored at the laboratory for approximately two years) and artificial sea water. The formula for artificial sea water was that of Zobell (1946). Several ingredients which were not available at the

laboratory were omitted. The complete formula consisted of:

NaCl	24.32 g	*SrCl ₂ .6H ₂	0.042 g
MgCl ₂ .6H ₂ O	10.99	H ₃ BO ₃	0.027
Na ₂ SO ₄	4.06	*Na ₂ SiO ₃ .9H ₂ O	0.005
CaCl ₂	1.14	*NaF	0.003
KCL	0.69	*NH ₄ NO ₃	0.002
NaHCO ₃	0.20	*FePO ₄ .4H ₂ O	0.001
*KBr	0.10	Dist. H ₂ O	1 liter

* Omitted

Following the initial experiments shown in Table 1, the term sea water refers to fresh sea water which had been filtered before use as a suspending medium. This was done to eliminate possible effects to other particulate mater present in sea water.

Phage Assay Method:

One ml volumes of the sample dilutions to be assayed were placed in sterile cotton plugged tubes (13mm x 100mm) held in a water bath at 45 C. One ml of overlay agar, previously melted and cooled at 45 C was added to each. Then 0.1 ml of a 5-6 hour broth culture of E. coli C2 was added and the tubes were shaken briefly in the water bath. The contents of the tubes were then poured over the surface of freshly prepared phage assay agar plates and the plates were rotated to spread the overlay. After the overlay had hardened, the plates were inverted and incubated at 28 C for 24 hours. Plaques were counted using a Quebec Colony Counter.

Filtration:

HA 0.45u and AA 0.8u filters¹ were used. Filtration apparatus included pyrex glass and stainless steel filter holders and a Swinny hypodermic adapter. The stock phage suspension was diluted 1:100 in the various preparations to be filtered. Final phage concentration in these preparations prior to filtration was usually 5×10^5 PFU/ml.

In some cases filters were treated by filtering 10 ml of various preparations prior to filtration of phage suspended in sea water. In elution experiments, 50 ml volumes of sea water containing 5×10^5 PFU/ml were filtered. A separate receiver was then placed under the filter apparatus and 50 ml of various elution media were filtered. The eluates were then assayed for phage content.

¹Millipore Filter Corp., Bedford, Mass.

RESULTS

The effects of several different suspending media on passage of phage through filters of two different pore sizes are shown in Table 1. In each case when phage was suspended in various types of sea water the percentage of phage recovered was very low. Percent recovered using the 0.8u filters was somewhat higher than when the 0.45 u type was used. When either distilled water or 0.1% peptone was used as the suspending medium, a high percentage of phage passed through the 0.45u filters. The disodium salt of ethylenediaminetetraacetic acid (EDTA), added in sufficient quantity to chelate the divalent cations in sea water, failed to improve phage passage. However, when peptone was added to natural sea water to a final concentration of 0.1% prior to filtration, 100% of the phage passed through the filter. To a lesser degree, an improvement in passage was also noted when this concentration of peptone was added to artificial sea water.

The effects of various filter components on phage filtration are shown in Table 2. Although some loss of phage occurred when the glass filter support was used, it is evident that the filter membrane was responsible for most of the loss.

The effects of several individual components of artificial sea water on filtration are shown in Table 3. The titrations of the unfiltered suspensions indicate that none of the individual chemicals or combinations tested were toxic. The results also indicate that there may have been a correlation between concentration of the individual chemicals and recovery of phage in the filtrate. Suspensions containing the two chemicals present in lowest concentrations, NaHCO_3 and H_3BO_3 showed the best phage recovery in the filtrates.

The effects of filter treatment on the passage of phage suspended in sea water are shown in Table 4. Filtration of 10 ml of either 1% or 5% peptone prior to filtration of phage suspended in sea water resulted in a high percentage of phage passage. No improvement was shown when chloroform was used for treatment. Results obtained using several methods for phage elution are shown in Table 5. The results indicate that only about 10% of the phage on the filter was eluted by any of the peptone concentrations used.

A few attempts at recovery of phage retained on the filter have also been made. Filters which had been used to filter phage-contaminated sea water were placed on agar and overlaid with agar and *E. coli* C2. Many plaques appeared following incubation. However, when attempts were made to apply this method quantitatively, the number of plaques found was much lower than the expected number.

DISCUSSION

Although the limiting pore size of the 0.45u filter is very nearly the same as the overall length of the phage, nearly complete recovery of the phage in the filtrates occurred when distilled water or 0.1% peptone water was used as the suspending medium. However, when suspended in sea water only about 10% of the phage passed through 0.8u filters. Even greater losses occurred when phage suspended in sea water was filtered through the 0.45u filter.

The results obtained using artificial sea water and individual chemical components of sea water indicate that the loss was not caused by some factor present only in natural sea water. The reversal of this effect when 0.1% peptone was either added to the sea water or filtered prior to sea water filtration indicated that some type of adsorption of phage particles onto the filter caused the losses. The addition of 0.1% peptone to artificial sea water also improved phage passage but to a lesser degree. Higher peptone concentrations, 1% or 5%, added to natural sea water also resulted in nearly 100% phage recovery. These higher concentrations were not tested in artificial sea water. The elution experiments, although not successful from the standpoint of total recovery, indicated that at least some of the phage was not destroyed by the adsorption process.

In a recent publication, Cliver (1965) reported somewhat similar results on filtration of enteroviruses suspended in various tissue culture media. Virus passage was dependent on suspending media even at filter porosities three or more times as large as the virus particle. Incorporation of serum into the suspending medium or treatment of filters with serum or gelatin prior to virus filtration resulted in marked improvement in virus passage.

In view of these results, the application of membrane filtration to the problem of detecting low levels of viruses in water appears promising. With better elution methods it may be possible to concentrate the viruses from a large volume sample into a much smaller volume of eluate with subsequent improvement in sensitivity of detection methods.

REFERENCES

- Cliver, D. O. 1965. Factors in the membrane filtration of enteroviruses. Appl. Microbiol. 13:417-425.
- Groman, N.B. and G. Suzuki. 1962. Temperature and lambda phage reproduction. J. Bacteriol. 84:431-437.
- Hoff, J. C. and W. J. Beck. 1964. Studies on the behavior of a bacteriophage in the Pacific oyster (Crassostrea gigas). Proc. N.W. Shell. Sanit. Res. Plann. Confer. PHS Pub. No. 999-FP-4.
- Hoff, J. C., W. Jakubowski, and W. J. Beck. 1965. Bacteriophage accumulation and elimination by the Pacific oyster (Crassostrea gigas). (Paper presented at Shellfish Sanit. Res. Conf., Ocean Shores, Wash.).
- Zobell, C. E. 1946. Marine microbiology. Chron. Bot. Co., Waltham, Mass. 240 p.

Table 1. Effects of filter pore size and suspending medium on filtration of *E. coli* C2 bacteriophage

Suspending Medium	AA 0.8u Filter		HA 0.45u Filter	
	PFU/ml:		PFU/ml:	
	in Unfiltered	Recovered in Filtrate	in Unfiltered	Recovered in Filtrate
Distilled Water	-	-	5.1×10^5	3.6×10^5
Distilled Water with 0.1% Peptone	6.4×10^5	5.3×10^5	4.2×10^5	3.2×10^5
Sea Water	5.0×10^5	6.9×10^4	4.4×10^5	2.3×10^3
Filtered Sea Water ^a	5.0×10^5	6.6×10^4	4.8×10^5	1.6×10^3
Filtered Sea Water ^a with EDTA ^b	-	-	3.5×10^5	$< 1 \times 10^2$
Filtered Sea Water ^a with 0.1% Peptone	-	-	5.2×10^5	5.2×10^5
Aged Sea Water (3 years)	-	-	6.6×10^5	2.3×10^3
Artificial Sea Water	-	-	6.6×10^5	1.4×10^3
Artificial Sea Water with 0.1% Peptone	-	-	4.6×10^5	9.0×10^4

^aFiltered through HA 0.45u filter before use as suspending medium.

^b165 MF/100 ml sea water.

Table 2. Effects of various filter components on filtration of phage suspended in artificial sea water

Filtration	PFU/ml:	
	In unfiltered	Recovered in Filtrate
HA 0.45u filter on glass filter support	5.9×10^5	1.2×10^3
Glass filter support only (no filter)		3.5×10^5
HA 0.45u filter on metal filter support		9.0×10^2
Metal filter support only (no filter)		5.1×10^5
HA 0.45u filter in Swinny adapter		6.6×10^3

Table 3. Effects of artificial sea water chemicals on phage filtration through 0.45u filter

Chemical	G/liter	mM	PFU/ml:		pH
			in Unfiltered	Recovered in Filtrate	
NaCl	24.32	415	4.8×10^5	2.0×10^2	5.6
MgCl ₂	10.99	115	6.6×10^5	3.0×10^2	5.6
Na ₂ SO ₄	4.06	28.6	5.6×10^5	1.0×10^2	6.0
CaCl ₂	1.14	10.2	3.7×10^5	1.4×10^3	6.4
KCl	0.69	9.3	5.1×10^5	6.0×10^2	5.7
NaHCO ₃	0.2	2.4	4.8×10^5	7.0×10^4	8.9
H ₃ BO ₃	0.027	0.44	5.8×10^5	3.3×10^5	5.8

Table 4. Effects of filter pretreatment on the passage of phage through HA 0.45u filters

Treatment ^a Medium	PFU/ml Recovered using Treated Filters ^b
Chloroform, U.S.P.	7.0×10^0
Distilled Water, with 0.1% Peptone	3.0×10^2
Distilled Water, with 1% Peptone	3.8×10^5
Distilled Water, with 5% Peptone	3.3×10^5

^aTreatment consisted of passing 10 ml of medium through filter before filtering sea water containing 4.6×10^5 PFU/ml.

^bBased on PFU/ml obtained after filtering 50 ml quantities of sea water containing 4.6×10^5 PFU/ml.

Table 5. Effect of Elution Media on the Recovery of Phage
From HA 0.45u Filters^a

Elution Medium ^b	PFU/ml Recovered in Eluate
Distilled water with 0.1% peptone	2.5×10^4
Distilled water with 1% peptone	2.0×10^4
Distilled water with 5% peptone	6.3×10^4

^a50 ml quantities of sea water containing 4.6×10^5 PFU/ml were passed through each of three HA 0.45u filters.

The filtrates contained 6.0×10^0 PFU/ml.

^b50 ml of each medium was used to elute the phage.

PART II
PROPOSED RESEARCH ACTIVITIES FOR
FISCAL YEAR 1966

PROPOSED RESEARCH ACTIVITIES

FOR FY 1966

W. J. Beck

INTRODUCTION

In the past this portion of our program has been of great importance to us. Your constructive criticism of our proposed projects has been helpful in directing our research programs. We hope you will continue with your valuable guidance on the proposals presented at this time.

During the past year we have completed several projects. These are now being prepared as technical reports or publications. Among the reports that should be ready within the next year are:

1. Bacteriological Studies on Storage of West Coast Shellfish.
2. Accumulation and Elimination of a Bacteriophage by Pacific Oysters.
3. Accumulation and Elimination of Bacteria by Hard-shell Clams.
4. Ecological Study of Bacterial Indices of Pollution.

We also plan to publish the proceedings of this Conference as was initiated last year.

PROPOSAL

Wet Storage of West Coast Shellfish

This project was suggested at the 1964 Research Planning Conference. The preliminary ground work has now been accomplished and the project should begin immediately. The project will be divided into two phases. The first phase will utilize controlled floats of shellfish located near the Northwest Shellfish Sanitation Laboratory. The second phase will be applied to commercial practices in the Puget Sound area. Only the first phase of the study will be initiated in FY 1966.

Pacific oysters and Manila clams will be harvested from the Burley Lagoon area. Floats will be loaded to various capacities. Determinations will be made on what effect this has on the shellfish as well as differences in bacteriological levels in various sections of the load. After loading the floats will be located so that the effects of tidal cycles and other physical factors can be studied. The floats will be held through four or more tidal cycles. During this period shellfish and water samples will be taken at regular intervals. Bacteriological tests will include coliform and fecal coliform tests as well as 20 C plate counts using McLeod Salt Water Medium. The study will be performed on a monthly basis in order to ascertain possible seasonal variations.

COMMENTS BY PARTICIPANTS

Drs. Chew and Sparks suggested the use of a thermograph for taking water temperature on a continuous basis at the float. This should be taken in addition to data collected at each sampling period.

Dr. Sparks and Mr. Beck discussed the use of graduate students to determine the physiological effect on the shellfish stored in the float.

A discussion was held by Messrs. Dupuy, McMillin, Beck and Dr. Sparks on how the clams should be held in the container. Several suggestions were made but it was decided to take Dr. Quayle's advice and not set up an artificial situation.

Dr. Quayle discussed the statistical design of the experiments and cautioned about adequate controls and replication during the project. Mr. Beck agreed that several preliminary experiments would have to be made before the experimental design could be completed.

Mr. McMillin inquired about location of the float, water samples to be taken and design of the float. Mr. Beck stated that the float would be placed initially in a tidalpool located in Burley Lagoon. Background data and concurrent water samples would be utilized in the data analysis. The float would have a screen bottom and sides to allow maximum exposure of the shellfish to the variance of the sea water.

Mr. Kelly summarized the project by indicating that the basic plan was to just determine the effect of the environment and then proceed into commercial practices.

PROPOSAL

Anaerobic Bacteriology

Studies will be continued on the incidence of Clostridium botulinum in estuarine areas. A series of samples of estuarine mud and related tributary stream soil samples will be tested to determine the incidence and persistence of C. botulinum in a given area. Estuarine mud samples will be taken at or near the head of the estuary. Attempts will be made to isolate and enumerate C. botulinum from associated salt water samples. In addition, samples will be collected from influent and effluent of a sewage treatment plant which empties into an estuarine area. Repeated samples will be taken throughout the year to determine residual incidence of C. botulinum.

Dry storage studies on clams artificial polluted with Type E C. botulinum will be made. Clams will be exposed to salt water containing approximately 100 spores per ml to determine accumulation, and possible outgrowth and toxin production within the body of the clam. Dry storage of the clam shellstock will be at 10 C and 20 C. Tests will be performed for toxin production. Methods of detection for multiplication of spores in shellfish will be investigated.

COMMENTS BY PARTICIPANTS

Dr. Sparks inquired about duplicate sampling from the same area at the same time. Mr. Beck reported that in the preliminary study distribution had been spotty and this would be considered in the proposal.

Mr. Jensen discussed the results reported by Dr. Eklund in that a difference in incidences was noted in different areas. Mr. Jensen asked if any plans were under way to consider these differences. Mr. Beck stated that other areas would be considered provided aid could be given in sample collection. Dr. Sparks and Mr. Blair volunteered to pick up samples from several areas in California and Oregon. The NWSSL is to provide sampling kits and make arrangements with the individual in order to avoid conflict with other activities.

Messrs. McMillin and Nelson requested information on the use of Manila clams in the accumulation-storage study. Mr. Beck stated that Manila clams would be the initial shellfish species studied but others would be used later. Where "gapers" are found they would be tested separately but with the same tests as that for the live shellfish. Dr. Eklund noted that the "gapers", toxin destruction may become apparent.

A discussion ensued between Messrs. Dupuy, Kelly and McMillin as to what storage temperatures should be used in the study. Mr. McMillin stated that his Company recommended immediate consumption for the aesthetic factor. It was agreed that the storage temperatures to be studied at this time would be -20, 10 and 20 C.

Dr. Eklund suggested that cooperative work between the two laboratories be considered. Mr. Beck agreed to divide certain samples and compare methodology between the Bureau of Commercial Fisheries Laboratory in Seattle and NWSSL.

PROPOSAL

Antimicrobial and Antiviral Agents in Shellfish

Testing of West Coast shellfish for the presence of antimicrobial agents will be continued. Antiviral tests will be included as techniques are developed and tissue cells are made available. Shellfish samples will be collected from the States of California, Oregon and Washington. Samples will be collected on a seasonal basis. Where antimicrobial and/or antiviral agents are found purification of the agents for further study will be attempted.

COMMENTS BY PARTICIPANTS

Dr. Liu and Mr. McMillin desired to know what the objectives of this study were and how much effort would be expended. Mr. Jensen replied that for once we should think positive about shellfish. Mr. Beck stated that the NWSSL could maintain a survey type project, but, could only expend a low number of man hours. Dr. Pringle agreed that at present there may be no commercial aspects apparent, but some investigation of the good things about shellfish should be done. Mr. Jensen noted that the original work by Dr. Li was also on a low priority order.

Dr. Liu agreed with the project and suggested the following: concentrate on the antiviral aspects as these products are not available today; a positive control such as an antibiotic should be included; dissection of organs should be performed before the purification is attempted; toxicity should be considered, if toxicity and activity are of the same level it is probably mostly a toxic effect; eggs should be used to study antiviral activity; the type of test virus is important, polio should not be used, tests against influenza or common cold viruses would be better.

It was agreed that this project should remain at a survey level for the present. If and when activity is found, a coordinated effort by the three shellfish sanitation centers would be advisable.

PROPOSAL

Effects of Ultraviolet Radiation on Enteroviruses in Sea Water

Review of the literature on this subject is now in progress. Studies on the inactivation of enteric viruses by ultraviolet radiation in sea water will be initiated. Factors such as salinity, temperature, and turbidity will be studied to determine the effect of each on rate of inactivation. The first test virus to be studied will be Polio Type I. In this manner background data for the development of the depuration process in removal of viral materials from West Coast species of shellfish can be obtained.

COMMENTS BY PARTICIPANTS

Mr. Timothy understood that this work had been accomplished and literature was available. Dr. Liu stated that Parke-Davis had done some work but no research had been done on viruses in sea water. He stated that about 170 viruses inhabit Eastern sea water and that every one of these viruses should be checked out against ultraviolet irradiation to be sure that the process is safe. Mr. McMillin stated that the primary objective of this project was to produce sterile water for depuration.

Mr. Kelly summarized the project by stating that most commercial and private institutions use the Purdy unit. Therefore, we need to know how effective this unit is against viruses and how to improve the system.

PROPOSAL

Pilot Model for Depuration of West Coast Clam Species

In 1964 discussions were held on the possibility of the establishment of a pilot plant study on the depuration of clams in the Puget Sound area. Due to mitigating circumstances this project was temporarily postponed.

A small salt water tank will be constructed at the Purdy site. The design of the tank will incorporate facilities for the recirculation and UV treatment of the salt water. The guide for this project will be that suggested in the forthcoming publication by S. A. Furfari, Interim Standard for Depuration Plant Design.

Studies will be made on the effects of various loading technics on the mortality and bacteriology of the clams used. Clams used in this study can be utilized in other studies at this station.

COMMENTS BY PARTICIPANTS

Mr. Felsing questioned the use of clams rather than oysters for the initial pilot plant. Mr. Dupuy agreed as there appeared to be more inherent problems with clams than with oysters. Mr. Girard reiterated an earlier statement that the problem in Washington would possibly be with clams rather than oysters. Mr. Beck explained the size of the facility that could be built in relation to the size of oysters and clams. Mr. McMillin asked about the relative problem with clams and oysters.

After some discussion the decision was made to start with clams, but, to proceed to the depuration of oysters as soon as possible.

PROPOSAL

Commensal Bacteriology

The objective of this investigation is to study the fate of different groups of microorganisms in live shellfish under dry storage conditions. Previously depurated shellfish will be allowed to accumulate the bacteria under study, and then be stored alive under dry storage conditions. By varying the number and types of bacterial species introduced, the amount of control exerted by the living animal on multiplication of different groups can be studied. In addition, antagonistic and synergistic effects of the bacterial species on one another can be studied.

It is anticipated that these studies will be carried out in cooperation with personnel of the College of Fisheries, University of Washington, Seattle, Washington.

COMMENTS BY PARTICIPANTS

Messrs. Dupuy and McMillin discussed the possibility of using sterile oysters which appeared to be difficult to obtain. Mr. Dupuy asked about studies on the outgrowth of organisms other than bacteria. Dr. Hoff replied that at the present time only bacteria could be considered.

Mr. Nelson inquired if an attempt would be made to correlate flavor quality with bacterial populations. Dr. Hoff replied that this was beyond the scope of the present facilities.

TECHNICAL ASSISTANCE PROGRAM

Several cooperative studies will be made in conjunction with the College of Fisheries, University of Washington, Seattle, Washington. It is anticipated that at least two studies by doctorate candidates at the College of Fisheries will be initiated within the next year.

One preliminary experiment has been conducted on the radiation of Pacific oyster shellstock. The oysters were artificially polluted with E. coli at the Purdy Station, radiated at the College of Fisheries, and returned for storage in flowing sea water to the Purdy Station. Results were not conclusive. Other cooperative experiments of this nature will be conducted.

In addition to the above, technical assistance will be given to the various states and Region as requested.

COMMENTS BY PARTICIPANTS

Dr. Liu and Mr. Beck discussed the limited results of one experiment using up to 100 kilorads on Pacific oyster shellstock. Reduction of no more than 50% of E. coli in the oyster should be investigated by repeating the experiment.

Mr. Jensen questioned the lack of pesticide examination on the West Coast. Mr. Beck stated that at the present no facilities or personnel are available. However, cooperative efforts have been made with Dr. Pringle at the Northeast Shellfish Sanitation Research Center.

At this point Dr. Pringle explained the problem in obtaining normal baselines for metals in shellfish. Standards used now were established 30 years ago. Dr. Pringle then reported on trace metals on the East Coast and a few samples obtained from middle and lower Puget Sound.

Mr. McMillin asked for consideration of an agent that could be added to the wash water to control bacterial growth. He would like to have a literature review on bactericidal agents used in other food products that might be applicable to oysters. Dr. Steers stated that the idea should be given some consideration but such an agent should not be used to cover up deterioration of the product. Mr. Jensen noted that this type of a study should be appropriate for the present laboratory.

As there was no more discussion on any of the topics presented the meeting was adjourned at 5:30 PM.

PART III

APPENDIX

AGENDA

NORTHWEST SHELLFISH SANITATION RESEARCH PLANNING CONFERENCE

September 20-21, 1965

Ocean Shores Inn

Ocean Shores, Washington

Monday Afternoon - Dan Quayle, Moderator

- 1:00 - 1:20 Opening Comments on Conference - Fred Kent
- 1:20 - 1:40 Summarization of Activities - W. J. Beck
- 1:40 - 2:00 Membrane Filter Technics as Applied to Estuarine and
Tributary Waters - W. J. Beck and G. J. Vasconcelos
- 2:00 - 2:15 Bacteriological Studies on Commercial Processing of
Olympia Oysters (Ostrea lurida) - G. J. Vasconcelos
- 2:15 - 2:40 Storage Studies on Shucked Olympia Oysters (Ostrea lurida)
- T. H. Ericksen
- 2:40 - 3:00 Storage Studies on Olympia Oyster Shellstock (Ostrea lurida)
- G. J. Vasconcelos
- 3:00 - 3:15 Coffee Break
- 3:15 - 3:30 Antimicrobial Agents in West Coast Shellfish - W. Jakubowski
- 3:30 - 4:00 Research Activities at other PHS Shellfish Sanitation Centers
C. B. Kelly, Chief, Research & Investigations Section
Shellfish Sanitation Branch, DEEFP, Washington, D. C.
- 4:00 - 4:20 Preliminary Ecological Studies on the Presence of C. botulinum
- T. H. Ericksen and W. J. Beck
- 4:20 - 5:00 Presence of C. botulinum on the West Coast - Melvin Eklund
Bureau of Commercial Fisheries, Department of Interior,
Seattle, Washington
- 6:00 - 7:00 No Host Reception
- 7:00 - 8:00 Dinner

Tuesday Morning - Edward Gruble, Moderator

9:00 - 9:30 Research in Virology at the PHS Shellfish Sanitation
Research Centers - Oscar Liu, Northeast Shellfish
Sanitation Research Center, Narragansett, Rhode Island

9:30 - 10:00 Studies on the Accumulation and Elimination of
Bacteriophage by Pacific oyster (Crassostrea gigas)
- J. C. Hoff, W. Jakubowski and W. J. Beck

10:00 - 10:30 Studies on Membrane Filtration of Bacteriophage
- J. C. Hoff and W. Jakubowski

10:30 - 10:45 Coffee Break

C. B. Kelly, Moderator

10:45 - 12:00 Research Proposals:
Wet Storage Studies
Ecological Studies on Anaerobic Bacteriology
Dry Storage Studies on C. botulinum

12:00 - 1:00 Lunch

1:00 - 5:00 Research Proposals (Cont'd.)

Antimicrobial Investigations
Virus Research
Depuration Studies
Commensal Bacteriology
Technical Assistance

5:00 - 5:15 Summary of Conference

ATTENDANCE ROSTER

Universities

D. Bevans	College of Fisheries, U of W.	Seattle, Washington
K. Chew	College of Fisheries, U of W.	Seattle, Washington
R. Digirolama	College of Fisheries, U of W.	Seattle, Washington
J. Dupuy	College of Fisheries, U of W.	Seattle, Washington
J. Matches	College of Fisheries, U of W.	Seattle, Washington
R. Neal	College of Fisheries, U of W.	Seattle, Washington
A. Sparks	College of Fisheries, U of W.	Seattle, Washington

State and Local Agencies

T. Blair	Oregon State Board of Health	Portland, Oregon
R. Cavanaugh	Seattle-King Co. Health Dept.	Seattle, Washington
H. Foster	Calif. State Dept. of Health	Berkeley, California
J. Girard	Wash. State Dept. of Health	Olympia, Washington
C. Hall	Wash. State Dept. of Health	Seattle, Washington
M. Hayes	Wash. State Dept. of Health	Olympia, Washington
K. Michener	Oregon State Board of Health	Portland, Oregon
R. Russell	Wash. State Dept. of Health	Olympia, Washington
C. Sayce	Wash. State Dept. of Fisheries	Nahcotta, Washington
C. Stonehouse	B. C. Provincial Health Dept.	Victoria, B. C.

Industry

E. Brenner	J. J. Brenner Oyster Company	Shelton, Washington
J. Christopherson	B. C. Packers	Vancouver, B. C.
E. Gruble	Hilton Seafoods Company	Seattle, Washington
D. McMillin	Olympia Oyster Company	Shelton, Washington
R. Murakami	Coast Oyster Company	South Bend, Washington
P. Parker	Pacific Coast Oyster Growers Assn.	Olympia, Washington
E. Timothy	Timothy Oyster Company	Ladysmith, B. C.

Federal Agencies

W. Beck	N.W. Shellfish Sanitation Lab.	Gig Harbor, Washington
W. Clothier	Pacific Northwest Water Lab.	Corvallis, Oregon
S. Copp	Canadian National Health Dept.	Vancouver, B. C.
M. Eklund	Bureau of Commercial Fisheries	Seattle, Washington
T. Ericksen	N.W. Shellfish Sanitation Lab.	Gig Harbor, Washington
W. Felsing	USDHEW PHS Region IX	San Francisco, Calif.
R. Garm	Department of Fisheries B. C.	Vancouver, B. C.
J. Hoff	N. W. Shellfish Sanitation Lab.	Gig Harbor, Washington
W. Jakubowski	N. W. Shellfish Sanitation Lab.	Gig Harbor, Washington

Federal Agencies (Continued)

E. Jensen	DEEFP PHS Shellfish San. Branch	Washington, D. C.
C. Kelly	DEEFP PHS Shellfish San. Branch	Washington, D. C.
O. Liu	Northeast Shellfish San. Res. Ctr.	Narragansett, R. I.
L. Myers	N.W. Shellfish Sanitation Lab.	Gig Harbor, Washington
D. Nelson	Bureau of Commercial Fisheries	Seattle, Washington
B. Pringle	Northeast Shellfish San. Res. Ctr.	Narragansett, R. I.
D. Quayle	Fisheries Research Board of Canada	Nanaimo, B. C.
A. Steers	Food and Drug Administration	Seattle, Washington
G. Vasconcelos	N.W. Shellfish Sanitation Lab.	Gig Harbor, Washington

U. S. GOVERNMENT PRINTING OFFICE : 1964 O-282-712